CLINICAL AND MOLECULAR ASPECTS OF ANAL HUMAN PAPILLOMAVIRUS, HUMAN PAPILLOMAVIRUS ASSOCIATED ANAL SQUAMOUS INTRAEPITHELIAL LESIONS, AND ANAL CANCER

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ABSTRACT

In the anal canal, human papillomavirus (HPV) infection is associated with the development of *Condyloma accuminata*, low-and high-grade dysplasia, and squamous carcinoma. In a population of men who have sex with men (MSM), anal HPV is common with 61% of HIV-negative and 93% of HIV-positive men testing positive for the virus using the polymerase chain reaction (PCR) technique. MSM also have high rates of anal dysplasia, the putative precursor of anal cancer. This lesion may be detected by anal cytology sampling and reported using Bethesda 2001 terminology. Definitive staging of anal dysplasia requires high-resolution anoscopy (HRA) with biopsy of lesions that are visually suspicious for dysplasia using criteria established for cervical colposcopy and validated for the anal canal. Data from the largest anal dysplasia natural history cohort report anal dysplasia rates of 7% in HIV-negative MSM, and 36% in HIV-positive MSM. In a population of MSM prior to the HIV-epidemic anal cancer rates were reported as 35 per 100,000 - similar to that of cervical cancer prior to routine cervical cytology screening in developed countries, and 35 times the rate of anal cancer in the general population. Currently, in a population of HIV-positive MSM, anal cancer rates are between 70-80 per 100,000 and unlike other HIV associated opportunistic infections and malignancies, show little if any response to highly active antiretroviral therapy (HAART).

The goal of this thesis is to describe clinical and molecular aspects of anal HPV and its' associated anal lesions, and has four main aims:

1. Identification of risk factors for the development of anal cancer: Eight subjects were identified from the UCSF pathology database with anal cancer and previously diagnosed high-grade anal dysplasia diagnosed by anal cytology or anal biopsy. Standard risk factors for anal cancer such as cigarette smoking and anal wart

diagnosis were each present in 5 individuals. However, compared to the general population, these subjects developed anal cancer at an earlier age (mean age 45.1 years with range 39-44 years), were predominantly male (7 out of 8), were predominantly HIV-positive (all males were diagnosed for at least 10 years and were all taking HAART), and had a short time from diagnosis of high-grade anal dysplasia to the development of anal cancer (average 14.4 months with a range of 3-23 months). These cases indicate that high-grade anal dysplasia may be a risk factor or marker for the subsequent development of anal cancer and that in the context of long-standing HIV infection, anal cancer may be diagnosed at an earlier age than in the general population.

2. Investigation of the efficacy of self-taken anal cytology swabs: No international or national standard exists for anal cytology testing and few medical centres provide this service. This study aimed to assess the efficacy of self-administered anal cytology sampling compared to clinician obtained specimens. 102 (82 HIV-positive and 20 HIV-negative) predominantly Caucasian MSM with previous paired anal cytology and anal biopsies completed the study. Satisfactory anal cytology specimens were obtained in 91% of self-collected samples and 99% of clinician-collected samples ($p=0.02$). The self-collected specimen adequacy rate was similar for HIV-positive (93%) and HIV-negative (85%) subjects ($p=0.37$). The sensitivity of self-collected and clinician-collected anal cytology to predict any grade of anal dysplasia was 68% and 70% respectively. However, HIV-positive subjects demonstrated higher sensitivity in detecting both cytological abnormality of any grade and high-grade cytological abnormality compared to HIV-negative subjects. Overall cytologic results did not differ by grade between clinician-collected and self-collected samples. These results suggest that a population of MSM with previous experience of anal cytology

testing and anal biopsy, with only written instructions, are capable of self-collecting samples with sensitivity comparable to experienced clinicians. In addition the sensitivity of anal cytology collected by clinicians and subjects to detect AIN 2 or 3 on biopsy was comparable. Thus self-collected anal cytology may allow high-risk populations to be screened outside of a medical setting.

3. Investigation of the feasibility of cytokine detection by reverse transcription PCR: The cytokine environment of the cervical canal in both health and disease states has previously been investigated. In particular, analyses have been performed using cervical cytological specimens and cervical biopsies from women with and without HPV associated cervical dysplasia. These finding tend to suggest that there is a down regulation of T helper (Th) type 1 cytokines, such as interferon gamma (IF-γ) and interleukin 2 (IL-2), that favour a cell mediated immune response compared to Th2 cytokines, such as Interleukin 4 (IL-4) and Interleukin 10 (IL-10), that favour a humoral immune response that is also associated with tumour progression. Such a Th1 to Th2 shift has also been described in the context of HIV/AIDS. In the context of anal dysplasia it was hypothesised that detection of a predominantly Th2 environment may be a marker for disease progression. The study aimed to detect IF-γ, IL-2, IL-4, and IL-10 by reverse transcription polymerase chain reaction (RT-PCR) of ribonucleic acid (RNA) derived from anal cytology specimens. Subsequently cytokine type would be related to the degree of anal dysplasia. Initial set-up experiments were performed to optimise parameters such as RNA isolation, PCR cycling, positive control sequence verification, quantification of lower limit of detection, magnesium and *Thermus aquaticus* concentration, and PCR machine function. However, likely due to the nature of the samples, RNA purity was low with resulting multiplicity of bands when PCR product was run on agarose gel. Southern transfer analysis of bands

likely to conform to two of the cytokines investigated showed no detectable signal. Likely causes for experimental failure include faecal contamination of specimens and low quantities of investigational cytokines in cytology specimens. Future experiments using RT-PCR or immuno-histochemical staining on fresh or fixed biopsy specimens respectively may be more likely to result in detectable cytokines, as has been reported from other gastrointestinal sites.

4. Investigation of the prevalence of age-related anal HPV and anal cytological abnormality, and their association with incident HIV infection in a cohort of HIV-negative MSM: Project EXPLORE was a two group randomized controlled phase IIb trial of a behavioural intervention to prevent HIV infection in a cohort of 4295 HIV-negative MSM. The HPV sub-study of EXPLORE was conducted in four cities in North America and aimed to characterise anal HPV and anal cytological changes in a wide age range of approximately 1200 HIV-negative MSM. Results showed a high anal HPV prevalence of 57% in all age ranges studied with a high-risk HPV prevalence of 26%, with HPV-16 the most commonly isolated type. Multiple anal HPV type infections were detected in 45% of participants. Risk factors for anal HPV detection were history of receptive anal intercourse and number of sex partners in the preceding 6 months. Anal cytological abnormalities were also common in this population with a prevalence of 20%. Risk factors identified for abnormal anal cytology were detection of anal HPV, detection of multiple HPV types, number of sex partners, number of receptive sex partners, injection drug use, and the use of amyl nitrate (poppers). This study illustrated for the first time the epidemiology of anal HPV infection and anal cytological diagnoses in a population of HIV-negative MSM recruited from a wide age-range. Additionally, detection of anal HPV and atypical anal cytology was associated with incident HIV infection.

For Ian, my parents, Patty,

and in memory of Joe Thatcher

DECLARATION

I declare that the contents of this thesis are my own work. Various members of the clinical and laboratory staff whose contributions have been noted in the acknowledgements section or in the text have assisted me in aspects of these studies. The work was carried out at the University of California San Francisco between 1998 and 2001.

Dr R. D. Cranston

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ABBREVIATIONS

AIDS	acquired immune deficiency syndrome
AIN	anal intraepithelial neoplasia
ASCH	atypical squamous cells suggestive of high-grade dysplasia
ASCUS	atypical squamous cells of undetermined significance
ASIL	anal squamous intraepithelial lesions
ATP	adenosine triphosphate
BCG	Bacille Calmette-Guerin
bp	base-pair
cDNA	complementary DNA
CDC	Centres for Disease Control
CIN	cervical intraepithelial neoplasia
CMV	cytomegalovirus
CSIL	cervical squamous intraepithelial lesion
CTL	cytotoxic T lymphocyte
dNTP	deoxynucleotide triphosphate
ECL	enhanced chemiluminescent detection
EDTA	ethylenediaminotetra-acetic acid
ELISA	enzyme-linked immunosorbent assay
FDA	Food and Drug Administration
g	gram
GAP-DH	glyceraldehyde-3-phosphate dehydrogenase
HC2	hybrid-capture 2
HIV	human immunodeficiency virus
HPV	human papillomavirus
HRA	high-resolution anoscopy
HSIL	high-grade squamous intraepithelial lesion
IF-γ	interferon gamma
IL-2	interleukin two
IL-4	interleukin four
IL-10	interleukin ten
IRC	infrared coagulator
kb	kilobase
LSIL	low-grade squamous intraepithelial lesion
M	molar
MA	milliamp
Mg	magnesium
Mϕ	macrophage
min	minute
MMLV	Maloney murine leukaemia virus
MSM	men who have sex with men
nm	nanometres
no	number
NaOH	sodium hydroxide
NK	natural killer
OC	oral contraceptive
OD	optical density
Pap	Papaniculaou
PBS	phosphate buffered saline

PCR	polymerase chain reaction
RT	reverse transcription
RT-PCR	reverse transcription polymerase chain reaction
s	second
SCC	squamous cell cancer
SD	standard deviation
SDS	sodium dodecyl sulphate
SE	standard error of the mean
SSC	standard sodium citrate
SSPE	saline, sodium phosphate, EDTA
TAE	Tris-Acetate-EDTA
Taq	*Thermus Acquaticus* DNA polymerase
Tm	melting temperature
Tris	tris[hydroxymethyl]aminomethane
U	unit
UCSF	University of California San Francisco
US	United States
V	Volt

PUBLICATIONS

ABSTRACTS

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CHAPTER 1

THE VIROLOGY OF HUMAN PAPILLOMAVIRUS

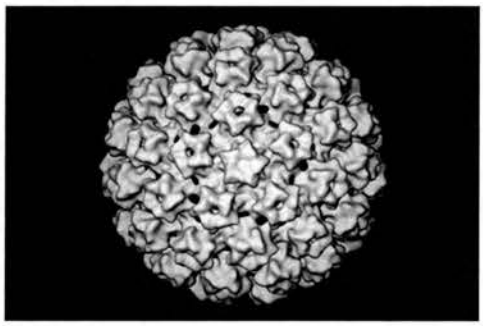
1.1 INTRODUCTION

This chapter will introduce the molecular biology of human papillomavirus (HPV) with emphasis on the function of HPV genomic products and their link to cell transformation.

1.2 HPV MORPHOLOGY

HPV is a non-enveloped icosohedral particle with a diameter of 55nm. The spherical viral shell is formed by 72 pentameric capsomers that enclose episomal double stranded DNA.(1) Both the capsid shell and enclosed HPV DNA are required to form an infectious viral particle. (See Figure 1.1)

Figure 1.1 Electron micrographic appearance of an HPV particle



1.3 HPV TROPISM

HPV infects stratified squamous epithelium of skin and mucous membranes. Some HPV types are epithelium-site specific, e.g. the HPV types that cause plantar warts are not detected in the oral cavity. Recent studies have shown that while HPV receptors are present and generally expressed on stratified squamous epithelium, viral replication may only occur in tissues that have intracellular regulators that facilitate type-specific HPV replication, thus explaining HPV's anatomical tropism.(2) While

this tropism is absolute for some HPV types, others such as HPV 16 may be found in both anogenital and oral locations.(3)

1.4 HPV ATTACHMENT AND INFECTION

While it was generally accepted that a receptor was necessary for HPV cell attachment and infection, until recently little was known about the cellular HPV receptor itself. In 1999 Joyce proposed that heparan sulphate proteoglycans (HSPG) function as universal HPV attachment receptors, that have since been shown to be essential for HPV internalisation for experimental virus like particles (VLP).(4) (5) (6) VLP are composed of self-assembled L1 proteins, without HPV DNA, that form the outer shell of infectious HPV.

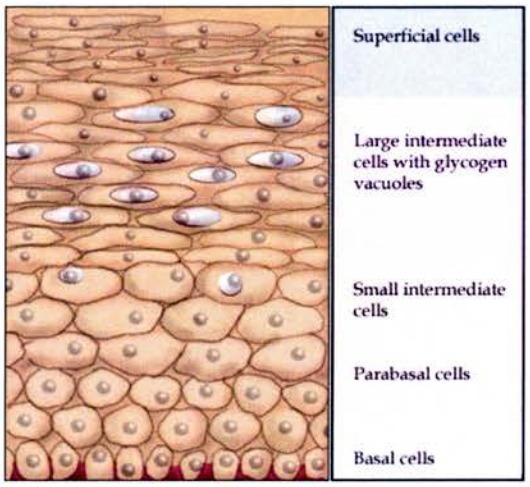
While HSPG appear to be ubiquitous, other authors suggest that other ligands such as alpha-6 integrin and as yet uncharacterised secondary receptors may also be involved in the infection process.(7) However, it is also worth noting that most HPV infection studies to date have used manufactured VLP that may or may not reflect natural infection.(7) Following attachment HPV internalisation has been shown to occur via clathrin-dependent receptor-mediated endocytosis.(8)

1.5 HPV REPLICATION

Following viral entry into the basal epithelial cell nucleus, HPV replicates by three methods that are dependent upon on the maturity and differentiation of the host cell. Squamous epithelium is arranged in five layers increasing in differentiation from basal, parabasal, small intermediate, large intermediate to superficial cells. (See Figure 1.2) Initially, plasmid replication occurs in the basal cell where HPV viral DNA is amplified to between 50-400 copies per diploid genome. Subsequently the

HPV genome replicates once per cell division as the epithelial cell matures, maintaining a constant HPV copy number per cell with equal division of genomic material to each daughter cell thus ensuring both latent and persistent infection. With maturation and terminal differentiation of the superficial layers of the epithelium, control of HPV replication is lost, and by an unknown mechanism, HPV replicates in an uncontrolled vegetative fashion. This replication, independent of cell division in the non-dividing cell, results in the formation of 1000's of copies of HPV per cell.

Figure 1.2: Cross section of normal squamous epithelium



1.6 VIRAL ASSEMBLY

Viral assembly occurs in the small intermediate and more superficial layers of the epithelium in terminally differentiated non-mitotically active cells where both early and late gene products are present.

1.7 HPV GENOME AND GENE PRODUCTS

HPV exists in the cell nucleus as a non-enveloped double-stranded DNA episome consisting of approximately 7,900 base pairs, and is associated with cellular histones to form a chromatin-like substance.(9) (10) The HPV genome encodes DNA sequences for six early (E) proteins associated with viral gene regulation and cell transformation, two late (L) proteins which form the protein shell of the virus, and a long control region (LCR) that contains regulatory sequences.(11) (12) In addition, there are two additional open reading frame's (ORF) whose function is unknown, E3 and E8. The ORF is a long DNA sequence that is uninterrupted by a stop codon and encodes part or all of a protein. (See Figure 1.3)

Following viral infection, HPV E gene expression can be demonstrated in the basal cells of the epithelium.(13) (14) Subsequently, L gene expression occurs but is restricted to terminally differentiated non-replicating cells of the epithelium.(15) It is this dependence on differentiating epithelia for full genomic expression and viral assembly that has hampered attempts to propagate HPV in the laboratory. Current methods of HPV propagation include inoculation under the renal capsule of nude mice, using a 'raft' culture system comprised of a stratified squamous epithelium at an air-water interface, or using monolayered cell cultures that can be modified to produce infectious viral particles. (16) (17) (18)

Figure 1.3: HPV open reading frame

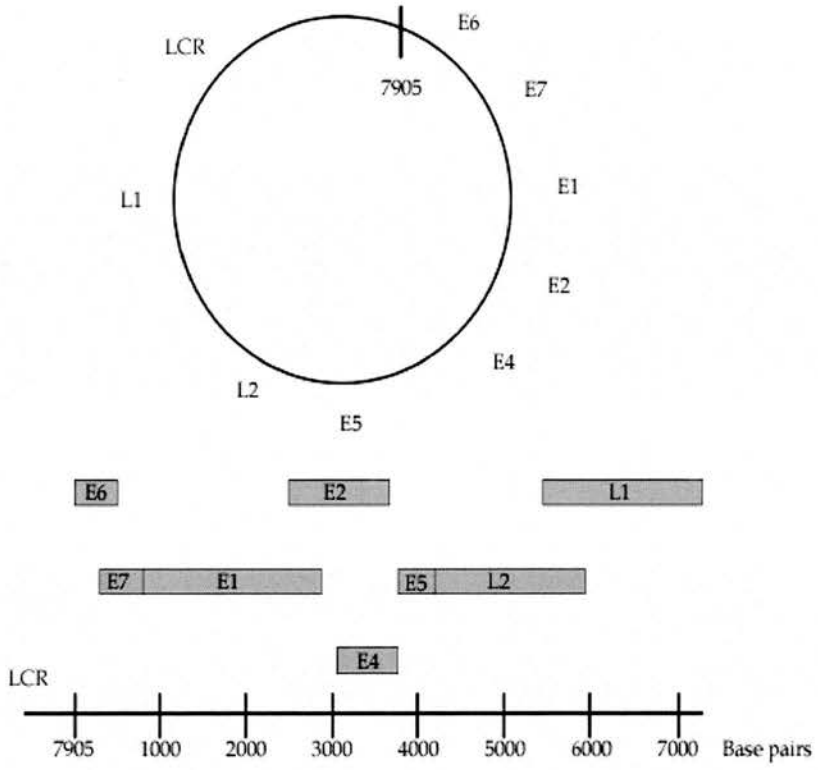


Image adapted from Fields (19)

1.7.1 E1

Both E1 and E2 are required for stable HPV replication. E1 maintains the viral episomal form, controls DNA unwinding prior to transcription, and subsequently initiates transcription with E2 acting as a cofactor, by the action of a helicase enzyme.(1) (20)

1.7.2 E2

E2 functions as a transcription regulator by binding to the origin of plasmid replication in addition to down-regulating expression of E6 and E7. Disruption at E2, which occurs after linearization and integration of the HPV episome into the host cell

genome, results in abrogation of E2 function and unchecked viral E6 and E7 genomic expression. This event is associated with development of a malignant cell phenotype. An integrated genome is more commonly demonstrated in high-grade dysplasia and squamous cell cancer than in low-grade dysplasia.(11)

1.7.3 E5

The role of E5 following HPV infection is poorly defined, but in high-risk HPV, E5 may speculatively be involved in the initiation and facilitation of the transformation process.(1) (21) E5 facilitates hyper-activation and increased recycling of epidermal derived growth factor (EDGF), enhancing the infected cells' response to growth factors and facilitating mitosis.(22) These E5 effects occur while the high-risk HPV is in the episomal form within the host cell nucleus. E5 is not expressed in HPV associated cancers and may be deleted following integration of the HPV episome into the host chromosome.(23) (24)

1.7.4 E6 and E7

There are numerous interconnected pathways involved in E6, E7 and cell cycle regulation and what follows are the most established mechanisms presented in the literature.

The two most important HPV proteins in the pathogenesis of malignant disease in high-risk HPVs are the oncoproteins E6 and E7. The continued expression of both these proteins is associated with the development of a malignant cell phenotype, although E7 alone *in vitro* is capable of transforming cells.(25) (26) (27)

The genotypic variations in the DNA base-sequences of E6 and E7 in different HPV types characterize the oncogenic phenotype of the virus into high- and low-risk types, with reference to their association with cervical cancer.(28) (29) (11)

At the molecular level, the ability of E6 and E7 proteins to transform cells relates in part to their interaction with two intracellular proteins, p53 and retinoblastoma (Rb).

1.7.4.1 E6

E6 protein from different HPVs form complexes with p53 with more (high-risk HPV) or less (low-risk HPV) avidity that correlates with the *in vivo* clinical behaviour and the *in vitro* transforming activity of these different papillomaviruses.(30)

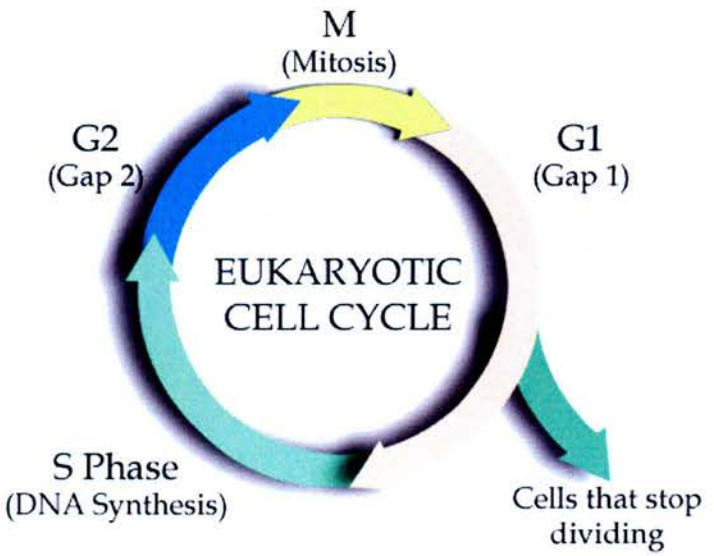
In the normal cell p53 is a negative regulator of cell growth that acts by inducing protein p21 expression. Protein 21 controls cell cycle transit from the resting (G1) to the synthetic (S) phase. (See Figure 1.4) It also functions as a tumour suppressor protein that halts cell growth following chromosomal damage, and allows either DNA repair enzymes to function, or induces apoptosis.(1) (31) (32) (33) (34) HPV E6 binds to E6 associated-protein (E6AP) leading to degradation of p53 by a ubiquitin dependent pathway that results in unchecked cellular cycling and the accumulation of chromosomal mutations without DNA repair. Other natural substrates of E6AP may also have an effect on E6AP function following E6 binding that may enhance or inhibit its effect on cell transformation. It is postulated that chromosomal mutations and genomic instability are required for HPV associated cell transformation and the development of a malignant phenotype.

1.7.4.2 E7

The Rb protein inhibits the effect of positive growth regulation and responds to DNA damage by halting cell growth and allowing DNA repair enzymes to work, or inducing cell apoptosis.(34) (35) One of the functions of Rb is to bind and render inactive the E2F transcription factor. E2F controls DNA synthesis and cyclin function and promotes the G1/S phase of cell cycling. (See Figure 1.4) E7 interacts with Rb protein in the E2F/Rb protein complex. When E7 binds to Rb protein, E2F is released and allows cyclin A to promote unregulated cell cycling.(23) (1, 36) These processes allow unchecked cell growth in the presence of genomic instability that may again lead to cell transformation, and the development of a malignant phenotype.

Rb changes phosphorylation status throughout the cell cycle, becoming phosphorylated and active in inhibiting cell cycling at the G1/S boundary and hypophosphorylated, without cell cycling inhibitory effects at late M. E7 binds to the hypophosphorylated form of Rb protein, thus the inactive stage is prolonged and cell cycling continues unchecked.(37) E7 binding can additionally lead to the destruction of Rb.(38)

Figure 1.4: The cell cycle



1.7.5 TELOMERES AND TELOMERASE

A telomere is a specialized structure made up of a recurring motif of 6 nucleotide bases (namely, the sequence TTAGGG) in addition to various other associated proteins involved in the replication and stability of the chromosome. Telomeres are found at the 5' end of eukaryotic chromosomes and function to protect the end of the DNA strand from destruction.(39, 40) Most cells do not have a mechanism for telomere repair and with successive mitoses the telomeres shorten until the cell undergoes p53 associated apoptosis.(41) However some cells such as germ cells have telomerase, a ribonucleoprotein enzyme complex (a cellular reverse transcriptase) in eukaryotic cells that can add telomeres to the ends of chromosomes after they divide, thus increasing the cells capacity for continued replication.(42)

In the context of HR-HPV infection, in addition to the effects of oncoproteins on p53 and Rb protein, HR-HPV E6 also activates telomerase. Telomerase is present in over 90% of anogenital cancers, it is present in cells immortalized *in vitro* by HPV E6, and is currently being investigated as a possible marker of high-grade lesions in cervical cytology samples.(43, 44)

1.7.6 L1 and L2

Both late proteins are expressed only in fully differentiated epithelial cells, and are therefore not expressed in high-grade dysplastic tissue or squamous cell cancer. L1 codes for the major capsid protein that induces both cellular and humoral immune responses and may be involved in viral cell attachment. L2 codes for the smaller minor capsid protein and has no currently defined function. Both L1 and L2 together, and L1 alone can self-assemble without viral DNA and form VLPs.(11) (45) This will be discussed further in the HPV vaccine section below.

1.7.7 LCR

The non-protein encoding LCR of HPV contains enhancer/promoter elements responsive to cellular factors as well as to virus encoded transcription factors. These enhancer elements are essential for the initial expression of viral genes.(11) (46) The LCR is responsible at least in part for the cell/tissue tropism of each HPV type, as the enhancer elements present in the LCR are tissue specific and involved in initiation of viral gene expression.(2)

Table 1.1 Summary of HPV gene product function and the LCR

HPV gene	HPV gene product function
E1	Maintains viral episomal form and controls and initiates transcription
E2	Transcription regulation and down-regulation of E6 and E7 expression
E5	Poorly defined but facilitates initiation of transformation in episomal HR-HPV by enhancing cell growth factor response
E6	Binds to E6 associated-protein leading to degradation of p53 resulting in unchecked cellular cycling
E7	Binds to Rb protein, E2F is released and allows cyclin A to promote unregulated cell cycling
L1/2	Form major and minor capsid proteins that encapsulate HPV DNA
LCR	Contains enhancer/promoter elements responsive to cellular factors and virus encoded transcription factors

1.8 GENOTYPIC CLASSIFICATION OF HPV

Recent molecular biological techniques using the polymerase chain reaction (PCR) and DNA probes have facilitated HPV typing and characterisation. Each HPV type is formally defined as unique by having less than 90 percent DNA nucleotide sequence homology with any another identified HPV type L1, E6 and E7 ORFs.(9) (47) There are currently over 110 types of HPV, with 38 of these displaying genitotropism.(48) (49) (50)

Within type HPV variants also exist that show 0-2% sequence heterogeneity in the L1, L2 and E6 genes.(51) These variants have ethnic and geographical correlations and show differing oncogenic potential in their association with dysplastic and malignant processes that may be related to virus infectivity, persistence, and ability to cause cell immortalisation or avoid immune surveillance.(52, 53) (54)

1.9 HPV PHENOTYPE

The association of HPV with malignant disease was first described by zur Hausen in 1976 with reference to cancer of the uterine cervix.(55) Lorincz subsequently suggested a HPV classification system based on this association using 3 categories: low-, intermediate-, and high-risk.(56) This classification has since been modified to high-risk types (HPV types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68) and low-risk types (6, 11, 42, 43, 44) only.(9) (57) Although helpful, this classification system is not absolute in that low-risk HPV may occasionally be found in cervical cancer tissue.

The phenotypic association of HPV and cervical dysplasia and cancer has also been demonstrated for HPV identified in anal dysplasia and anal cancer tissue. HPV 16 was the most common HPV type isolated from pathological specimens in a cohort of men

and women with an anal cancer diagnosis.(58) In 1997 Palefsky *et al* reported that high-risk HPV detected by Hybrid Capture (HC) was a risk factor for abnormal anal cytology in a population of men who have sex with men (MSM) with Centres for Disease Control (CDC) group IV HIV disease – an association subsequently confirmed by other authors.(59) (60) (61) Williams *et al* had previously reported the association of abnormal anal cytology with HPV infection detected by dot blot in a cohort of HIV-positive and HIV-negative women.(62)

1.10 TRANSFORMATION CO-FACTORS

In the female cervix, HPV is required but is not adequate for malignant transformation that only occurs when oncogenic co-factors are present. Co-factors implicated in the development of cervical squamous cancer include cigarette smoking, and possibly also prior infection with *Chlamydia trachomatis*. (63) (64) (65) (66) (67) It is also possible that cigarette smoking may be a co-factor in the development of anal cancer.(68)

Recently a large epidemiology study addressing the development of cervical cancer in women reported that among HPV positive women, high parity, and long-term oral contraceptive (OC) use in addition to smoking and co-infection with other sexually transmitted agents are consistently identified as environmental co-factors that increase the risk of progression from cervical HPV infection to high-grade squamous intraepithelial lesions (HSIL) and invasive cervical cancer. The authors also noted that there is limited evidence for a role of dietary factors in HPV carcinogenesis.(69)

1.11 SUMMARY OF SQUAMOUS CELL TRANSFORMATION

Following epithelial basal cell infection with HR-HPV and viral replication, the nuclear episome may linearise and become integrated into the host cell genome. This fracture within the viral E1 and E2 ORFs critically disrupts the suppressor functions of E2 with resulting uncontrolled expression of E6 and E7 oncoproteins. Uncontrolled cell cycling in the presence of telomerase occurs with resulting disruption of DNA repair mechanisms and accumulation of mutated DNA that may facilitate cell transformation, clonal expansion, and tumour formation.

CHAPTER 2

CLINICAL HUMAN PAPILLOMAVIRUS INFECTION

2.1 INTRODUCTION

This chapter will describe HPV infection, HPV/HIV co-infection, HPV detection, and different modalities of HPV and HPV associated lesion treatment with particular reference to the anal canal.

2.2 HPV INFECTION

HPV infects keratinizing and non-keratinizing stratified squamous epithelia and elicits a wide range of tissue responses ranging from histological latency to benign proliferative disease to invasive squamous cancer in the presence of co-factors.(1) (70)

HPV infection is commonly asymptomatic, but may cause itch and burning and is usually diagnosed clinically by the presence of clinical manifestations such as exophytic lesions, or subclinically in the form of abnormal cytology.(71) However it is now possible with molecular techniques such as Hybrid Capture (HC) and the polymerase chain reaction (PCR) (see below) to diagnose HPV infection *per se* for investigation purposes and clinical management, such as currently occurs in the triage of cervical cytology that shows atypical squamous cells of undetermined significance (ASCUS). However, diagnosing HPV in subjects without clinical evidence of infection has also been shown to negatively impact the individual's psychological well being and many subjects variously react with anger and distress upon learning of their infection indicating a sexually transmitted infection, as occurs with a diagnosis of genital warts.(72, 73)

2.3 HPV TRANSMISSION

Genitotropic HPVs are transmitted by close physical contact including sexual contact. The epidemiological evidence for this was derived from studies showing an increasing prevalence of HPV infection with increasing numbers of sexual partners, partners of individuals with genital warts subsequently developing genital warts, and concordance of HPV types between partners.(74) (75) (76) (77) (78) The rate of HPV transmission per sexual contact is unknown, but given the high prevalence of HPV infection in the general population it is likely to be high.

2.4 HPV IN THE GENERAL POPULATION

Warts were first associated with a viral cause by Ciuffo in 1907, with a viral cytopathic effect in 1968, and with HPV in 1981.(79) HPV is the most common viral sexually transmitted infection in the United States (US) with an increasing incidence, as in all developed countries, since the 1950's.(80) It is estimated that the majority of the sexually active adult population in the US have been infected with a genitotropic HPV based on antibody studies although only 1% (approximately 1.4 million persons) display clinical genital warts.(81) Sub-clinical HPV infections may be detected by colposcopy and cytology, and increasingly by molecular techniques.(82) (83)

2.5 EPIDEMIOLOGY OF HPV INFECTION

In a random sample of women attending primary care clinics, a seven-fold increase in the detection of clinically diagnosed genital warts was seen between 1966 and 1984.(80) Cytological evidence of HPV infection ranges between 8 and 24% depending upon the population sampled, with highest infections prevalence in women

under 25 years of age.(84) (85) (82) Cervical HPV infection prevalence rises rapidly after sexual debut and then decreases over subsequent years.

2.6 HPV AND IMMUNOSUPPRESSION

Immunosuppression *per se*, whether by HIV or induced iatrogenically in the context of solid organ transplantation, is associated with more frequent isolation of HPV, higher detectable HPV DNA levels, and the more frequent detection of multiple HPV types.(86) (87) (88) There is also a higher burden of clinical disease related to HPV infection in this population.(89-91)

2.7 HPV/HIV CO-INFECTION

HPV associated clinical disease occurs more commonly in the context of HIV infection. Women with HIV infection are at increased risk for cervicovaginal HPV infection and cervical intraepithelial neoplasia (CIN), and in 1993 cervical carcinoma was added to the list of AIDS defining conditions by the Centres for Disease Control (CDC).(92) (93) The effect of HAART has been investigated in women and has not been shown to affect HPV detection.(94) (95) However, data on the effect of HAART on the natural history of cervical dysplasia are conflicted with some studies showing no effect of HAART,(95, 96) and others show a statistically significant but modest beneficial effect.(94) (97) HIV infected MSM are at higher risk of HPV infection of the penile coronal sulcus, and are more likely to have capsid antibodies to high-risk HPV (but not low-risk HPV) than HIV-negative MSM. (98) (99) Anal HPV infection in the context of HIV will be discussed below.

2.7.1 HPV/HIV INTERACTION

HPV associated disease such as condylamata, anogenital dysplasias and anogenital cancer is more commonly diagnosed in HIV infected individuals.(100) (12) The possible mechanisms of pathogenesis include impairment of cell mediated immune (CMI) responses, epithelial cytokine dysregulation, and a direct effect of HIV proteins such as *tat* on HPV replication.(101) It is theoretically feasible that one or all of these mechanisms may also contribute to the increased incidence of anal cancer seen in an HIV-infected population.

2.8 ANAL HPV

Most studies of anal HPV have been undertaken in MSM. The spectrum of HPV types in the anal canal is similar to that described in the cervix and is associated with the same 'risk' phenotypes. HPV 16 is the most commonly detected HPV type associated with anal dysplasia and cancer, as well as cervical dysplasia and cancer.(102) (57) (103) (58) However, a few of the more commonly isolated HPV types in the anal samples have only rarely been reported in cervical samples (types 53, 58, 61, 70). As a group, these types show homology with HPV 39, which is high-risk in its association with cervical cancer.(104) HPV 32, characteristically an oral HPV type, has also been isolated in anal samples and may indicate transmission by oral-anal intercourse.(102)

The range of anal HPV types detected is similar in both HIV-positive and HIV-negative MSM with HIV-positive MSM more frequently diagnosed with multiple types, have a higher prevalence of HPV, and higher levels of high-risk HPV when semi-quantitative methods of detection are used.(102) (105)

2.8.1 RISK FACTORS FOR ANAL HPV

HPV may be detected in the epithelium of the penis, scrotum, anal canal, cervix, vagina, vulva and peri-anal area. Genital HPV infections are spread by penetrative sex and close physical contact involving an infected area, (86) however digital/anal, oral/anal and digital/vaginal contact are also likely to spread the virus as may fomites such as sex toys.(86) Risk factors reported for anal HPV infection are shown in Table 2.1.

Table 2.1 Risk-factors for anal HPV infection

History of receptive anal intercourse (102) (106)
History of anal warts (107)
Younger age (108)
HIV infection (109) (107) (61) (110)
HIV infection with a lower CD4 count (102) (109) (110)
History of smoking tobacco (109)
Recreational rectal drug use (102)

2.8.2 DEMOGRAPHICS OF ANAL HPV

No comprehensive information is currently available on the demographics of anal HPV in the general US population as HPV is not a notifiable sexually transmitted infection.(82) Centres investigating anal HPV most commonly have study populations of MSM who tend to be between the ages of 30 and 50 years, live in urban American centres, and have a high prevalence of HIV infection. However, a recent study has reported a high prevalence of anal HPV infection in a population of HIV-negative

urban MSM. This 4 site study of 1218 men between 18 and 89 years of age revealed a near constant prevalence of 57% in all age ranges studied with no significant differences noted in prevalence between urban centres.(111) (See Appendix 4)

2.8.3 ANAL HIV/HPV

In the context of HIV co-infection, most anal HPV research has been conducted in MSM. Using PCR detection methods, one group identified 29 individual types and 10 HPV groups from the anal canal of MSM with concomitant HIV infection.(102) Additionally, HIV-positive individuals with reduced CD4 counts have an over representation of high-risk HPV,(112) (102) increased transcription of early HPV genes,(112) and are more likely to shed HIV RNA from the anal canal in association with detectable HPV DNA.(113)

In women, data from the Women's Interagency HIV Study showed that 76% of 223 HIV-positive women were found to have anal HPV infection, as did 42% of high-risk (commercial sex workers and injection drug users) HIV-negative women.(114) Anal HPV infection appears to be at least as common as cervical HPV infection in HIV-positive women,(62) with one study showing that anal infection was more common than cervical infection, with two site concordance of HPV types reported as 50%.(115)

In adolescent women with HIV immunosuppression, as measured by reduced CD4 count, HIV was not a risk for HPV detection.(116)

2.8.4 TREATMENT OF ANAL HPV ASSOCIATED LESIONS

There is no current Food and Drug Administration (FDA) approved antiviral drug with activity against HPV. However, therapeutic vaccines directed against HPV-

infected cells are being explored as is prophylactic vaccination.(117) (118) (119) (120) (121) The following treatment modalities have been used to treat anal HPV associated lesions. Treatment of anal high-grade dysplastic lesions will be considered in the following chapter.

2.8.4.1 PODOPHYLLIN/PODOPHYLLOTOXIN

Podophyllin is an extract of the rhizome of *Podophyllum peltatum*, commonly known as American mandrake. (See Figure 2.1) It contains the antimitotic agent podophyllotoxin, which arrests the cell cycle in metaphase and leads to cell death. Podophyllin solution when used topically as a single agent once or twice a week has limited success in clearing warts ranging from 20 to 50 percent at three months.(122) It is usually employed as a 25 percent solution in combination with another treatment method such as cryotherapy. The drug is teratogenic and must not be used in pregnancy or suspected pregnancy and it is not indicated for internal use. Adverse effects range from mild skin irritation to ulceration and pain depending upon the concentration used and the length of time over which it is applied to the skin before removal by washing.

More recently, podophyllotoxin, the active element of podophyllin has been available as a solution or cream in concentrations of 0.15-0.5 percent. This preparation has the advantage of less clinical adverse events,(123) (124) consistency of formulation,(125) and improved efficacy when compared to podophyllin therapy.(126) (127)

Figure 2.1: American mandrake (*Podophyllum peltatum*)

2.8.4.2 TRICHLOROACETIC ACID

Eighty percent trichloroacetic acid (TCA) physically destroys wart tissue by protein coagulation. Clearance rates and adverse effects are similar to podophyllin and repeated application may be required. In contrast to podophyllin TCA can be used for internal lesions and in pregnancy.(128)

2.8.4.3 CRYOTHERAPY

Cryotherapy is performed by the application of liquid nitrogen spray, a swab soaked in liquid nitrogen, or by placing a nitrous oxide cooled cryoprobe on the lesion to be treated. This procedure is safe in pregnancy. Subjects usually experience mild pain during application and a variable degree of localized inflammation afterwards. Clearance rates at three months are 63 to 92 percent and again repeated application may be required.(128)

2.8.4.4 CO_2 LASER

Carbon dioxide laser therapy is carried out in an operating theatre and requires anesthesia. The HPV infected tissue absorbs the laser energy, which is converted into heat energy and the lesion is vaporized. This technique has rates of wart clearance that approach 100 percent over one year; however, recurrences can be up to 45 percent.(128) (129) Adverse events include scarring and pain.(130) It should be noted that laser surgery of condylomata has been associated with laryngeal papillomatosis developing in the operating surgeon.(131)

2.8.4.5 SURGERY

Surgical therapy may be considered when medical therapy has failed, when warts are very large, or are amenable to surgical removal. Scissor excision requires anesthesia and routine surgical risks such as infection and hemorrhage apply. The three-month clearance rates are 36 percent.(128) Circumferential anal lesions are best treated in two stages to reduce the risk of anal stricture and stenosis.

2.8.4.6 CIDOFOVIR

Cidofovir is an acyclic nucleotide analogue active against DNA viruses and licensed for the treatment of cytomegalovirus (CMV) infection. It has shown clinical efficacy in the treatment of external genital warts related to low-risk HPV infection,(132) supporting previous *in vitro* evidence of the growth inhibitory effects of cidofovir on HPV-positive cells.(133) (134) Cidofovir is not currently licensed for the treatment of HPV related disease.

2.8.4.7 INFRARED COAGULATION

This device is used in an outpatient setting and delivers infrared light in aliquots of 0.5 to 3 second intervals to previously anesthetized epithelium. The Infrared coagulator (IRC) produces a focal shallow burn in millimeter depth that correspond to the length of the light aliquot in seconds.(135, 136) The coagulated tissue is then removed by debridement or by excision using baby Tischler forceps. Small studies with encouraging results and response rates of 70% have reported to date.(137)

2.8.4.8 TOPICAL PHOTODYNAMIC THERAPY

Topical photodymamic therapy (PDT) employs the use of a photosensitizing compound, such as 5-aminolaevulinic acid - a precursor in the heme biosynthetic pathway, followed by subsequent exposure to a light source. The mechanism of action relates to the release of highly reactive oxygen species with local tissue destruction and inflammation. In one study 5-aminolaevulinic acid was used to treat urethral warts with response rates of 95% at 6-24 months following the procedure.(138)

2.8.4.9 IMIQUIMOD

Imiquimod is a non-nucleoside heterocyclic amine and Toll-like receptor 7 agonist. It is classified as an immune response modifier due to its ability to stimulate production of cytokines such as interferon-gamma, tumor necrosis factor-alpha and interleukin-6 in monocytes/macrophages thus creating a T helper (Th) 1 like environment and promoting a cell mediated immune response.(139) (140) Application of Imiquimod as a 5% cream three times per week has shown efficacy in the treatment of external anogenital warts with reduced recurrence rates compared with other modalities.(141, 142)

2.8.4.10 INTERFERON

Inferferons are a multigene family of proteins that can be divided into alpha (IF-α), beta (IF-β), and gamma (IF-γ), all of which have antiviral and immunoregulatory properties which they exert by binding to cell surface receptors.(143) Interferons have been used in HPV infection, usually by intralesional injection to genital warts although they may be applied topically or given systemically, with proven efficacy.(144) (145) The limiting factor for this therapy is high cost, and associated flu-like symptoms.

2.8.4.11 GENE THERAPY

As HPV associated cancers rely on continuous presence of E6 and E7 proteins it is theoretically possible that by abrogating the expression of E6 and E7 genes that the malignant phenotype of the cell may be lost. Potential mechanisms for this include use of antisense RNA, ribozymes, or short interfering RNA.(146)

2.8.4.12 HPV VACCINES

Both prophylactic and therapeutic vaccines are in Phase 3 clinical trials for the prevention and treatment of HPV associated lesions respectively.

2.8.4.12.1 THERAPEUTIC HPV VACCINATION

It is apparent that cell mediated immune responses are involved in the control of HPV infection as demonstrated by the increased frequency of clinically apparent warts and cervical/anal dysplasia in immunosuppressed individuals,(147) (148) (149) (150) the presence of lymphocytic infiltrates in biopsy specimens of cervical tumour

tissue,(151) and the presence of specific cytotoxic T lymphocyte (CTL) responses to HPV viral epitopes.(120) Thus, therapeutic vaccines aim to augment the established host cell-mediated immunological response and mount an enhanced, specific and directed T-cell mediated attack on the HPV-infected cell with down-regulation of viral reproduction or even viral clearance. Challenges in HPV associated cancer vaccine development are compounded by the down-regulation of major histocompatability class 1 (MHC1) antigen on tumour cells restricting opportunity for antigen presentation, and the development of immune tolerance.(152)

The therapeutic vaccine preparation methods to produce a directed cell-mediated response vary and include:

- Adaptive cellular transfer. This method involves collecting immune system cells from a subject, or a histocompatible donor with HPV infection and stimulating the cells *ex vivo* with recombinant IL-2. The cells, thus activated, are returned to the donor. This method has been used in murine HPV-positive tumour models with some effect.(120)
- Fusing HPV peptides to immunogenic moieties to enhance the existing immune response. Heat shock proteins acting as natural adjuvants have been shown to induce CTLs.(152) Phase 3 clinical trials are currently recruiting using the E7 protein of HPV 16 fused to a Bacille Calmette-Guerin (BCG) heatshock protein, Hsp 65, as this has shown promise in a murine model of squamous cancer.(153)
- Expressing HPV 16 and 18 E6 and E7 epitope peptides in a recombinant vaccinia viral particle. This method has been investigated in humans with advanced cervical cancer with a demonstrable effector CTL response in one out of eight patients, and no significant reported toxicity.(121)

- DNA vaccine. One Phase 1 clinical trial has reported the use of plasmid DNA encoding for HLA-A2 restricted epitopes derived from HPV-16 E7 protein, encapsulated in a biodegradable polymer, and formulated into microparticles. Study subjects were HPV-16 positive with high-grade anal dysplasia and an HLA-A2 haplotype. The vaccine was well tolerated and histological responses were noted in 3 of 12 eligible subjects.(154)

2.8.4.12.2 PROPHYLACTIC HPV VACCINATION

Prophylactic HPV vaccines contain HPV sub-units that induce neutralizing antibody prior to the host encountering natural HPV infection. The epitopes used in these vaccines are the major capsid proteins of HPV (L1 alone or in combination with L2), which self-assemble into virus-like particles (VLP) with no enclosed DNA, and have shown effect in animal models with concurrent induction of a CMI response.(155) (34) The challenges of this vaccine are the potential that only type-specific restricted responses are induced by each VLP.(118) Thus, to provide protection against the occurrence of warts as well as anogenital dysplasia, L1 capsid VLP from both high- and low-risk HPV are being considered.

Koutsky *et al* reported findings from an HPV 16 L1 virus like particle (VLP) vaccine given in three intramuscular doses over six months to sexually active females not previously exposed to HPV16 (negative cervical DNA analysis/cytology). The vaccine efficacy was 100% in preventing HPV 16 and HPV 16-related cytological abnormality.(156) Subsequently clinical trials have been performed with low- and high-risk bivalent VLPs that have showed long lasting antibody responses.(157) Furthermore a Phase 2 clinical trial reported high efficacy with a bivalent HPV 16 and 18 VLP vaccine in women.(158) While it is biologically plausible that these

promising results will also occur in men, no prophylactic vaccine trial has yet reported in this population.

There is no current FDA approved HPV prophylactic or therapeutic vaccine available for clinical use. However this is likely to change in the near future when issues such as age at time of vaccination, and pre-testing of minors for a sexually transmitted infection will need to be addressed. This is particularly relevant given the high reported rates of serological evidence of high-risk anogenital HPV infection in this population that may potentially compromise vaccine efficacy.(159)

2.9 HPV DETECTION

There are a number of clinical and molecular methods of HPV detection.

2.9.1 VISUAL INSPECTION

HPV associated lesions may have a condylomatous, flat, papular or keratotic appearance on the anogenital epithelium, or may be undetectable to the naked eye. (See Figure 2.2)

Figure 2.2: Perianal condylomata

Image courtesy of UCSF Anal Neoplasia Study

2.9.2 COLPOSCOPY

Colposcopy employs a binocular or video microscope with a focal length of between 300-350 mm that allows the clinician to observe the anogenital epithelium at magnification. The magnifications commonly used are 10, 16 and 25 times normal. HPV associated genital warts and high-grade dysplastic tissue has well-defined appearances in the cervix that are similar to those present in the anal canal.(160) (161) In addition to magnification, the use of stains such as 3% acetic acid and Lugols Iodine are employed to better define lesions. Acetic acid characteristically causes transient 'aceto-whitening' of abnormal epithelia, and despite being a non-specific change, one study demonstrated the presence of HPV using the Hybrid Capture 2 (HC2) detection system in more that 85% of acetowhite cervical lesions.(162) Lugol's iodine is taken up by normal glycogenated cells but not by dysplastic cells with reduced glycogen content, and may be used to confirm clinical suspicion of dysplastic tissue.(163) The use of the colposcope (high resolution anoscope (HRA) in the context of anal disease) is user dependent and requires training and the development of expertise prior to use. While no formal training exists for HRA, the American Society for Cervical Colposcopy oversees accreditation of cervical colposcopists in the US.

2.9.3 CYTOLOGY

Exfoliative cervical cytology has been employed since the 1950's in industrialized countries to detect and predict the severity of cervical dysplasia before HPV was demonstrated to be the etiological agent.(164) This method will be further discussed in Chapter 3.

2.9.4 SEROLOGY

Human serum has antibodies that react with HPV proteins.(165) Viral antigens in the form of VLPs using L1 expressed in eukaryotic systems have been produced that, in enzyme-linked immunosorbent assays (ELISA), show type-specific antibodies that correlate with a subject's clinical history of HPV infection.(166) (167) (168) The time interval between infection and seropositivity has been shown to vary by HPV type in a cohort of university women with incident HPV infections, 59.5%, 54.1%, and 68.8% seroconverted for HPV 16, 18, or 6, respectively within 18 months of detecting corresponding cervical HPV DNA.(169) It is unknown how long the HPV antibodies remains detectable. Using antibody detection, investigators have reported that more than 75% of women in the US are/will be exposed to anogenital HPV in their lives with most infections occurring with high-risk HPV (HPV-16).(170) (171)

2.9.5 HPV DNA DETECTION

There are several methods available to detect the presence and type of HPV DNA including in-situ hybridization (ISH), Southern transfer hybridization (STH), Hybrid Capture (HC), dot blot (DB) and filter hybridisation (FH), and PCR.(172) HC provides a semi-quantitative measure of HPV by the production of a chemiluminescent signal, while more recently quantitative real-time PCR can also be used to measure DNA levels.(173)

These tests can also be grouped according to their method of HPV DNA detection: PCR by target amplification, HC by signal amplification, and ISH/FH/DB/STH require no amplification.

2.9.5.1 IN-SITU HYBRIDISATION (ISH)

The advantage of ISH is that it is performed directly on tissue with specific DNA probes applied to de-natured DNA in permeabilized cells. Probes containing radiolabelled nucleotides can then be detected following exposure to photographic film. The process can also be performed with non-radiolabelled techniques, e.g., biotin. This process allows localisation of the target DNA sequences and correlation with the cytopathological appearance of the tissue. This method requires five or ten HPV genomes per cell to give a positive result, is less sensitive than PCR or HC, and is more labor intensive. However, when augmented by PCR, the sensitivity can be increased to one HPV genome copy per cell. There is currently no clinical indication for the routine use of ISH.(172)

2.9.5.2 DOT BLOT AND SOUTHERN TRANSFER HYBRIDISATION

DB, FH, and STH all require binding, by hydrogen bonding and hydrophobic interactions, of denatured target DNA to filter supports prior to blocking to prevent non-specific binding. The target is then hybridized with a complimentary labeled DNA probe, washed and then detected by differing methods depending on the probe label used. STH differs from the other methods by the addition of an electrophoretic separation step that increases the specificity of the test and may use restriction endonucleases for each HPV type before applying the DNA probe. All of these tests produce qualitative results and are occasionally used in research settings but are not used in clinical practice.(172)

2.9.5.3 FILTER IN-SITU HYBRIDISATION

This method combines aspects of both in-situ and dot blot hybridization with the application of cells onto filters where they are treated to denature the DNA, then hybridized and signals generated as for ISH. This method has poor sensitivity and specificity and is of methodological interest only.(174)

2.9.5.4 HYBRID CAPTURE (SOLUTION HYBRIDISATION)

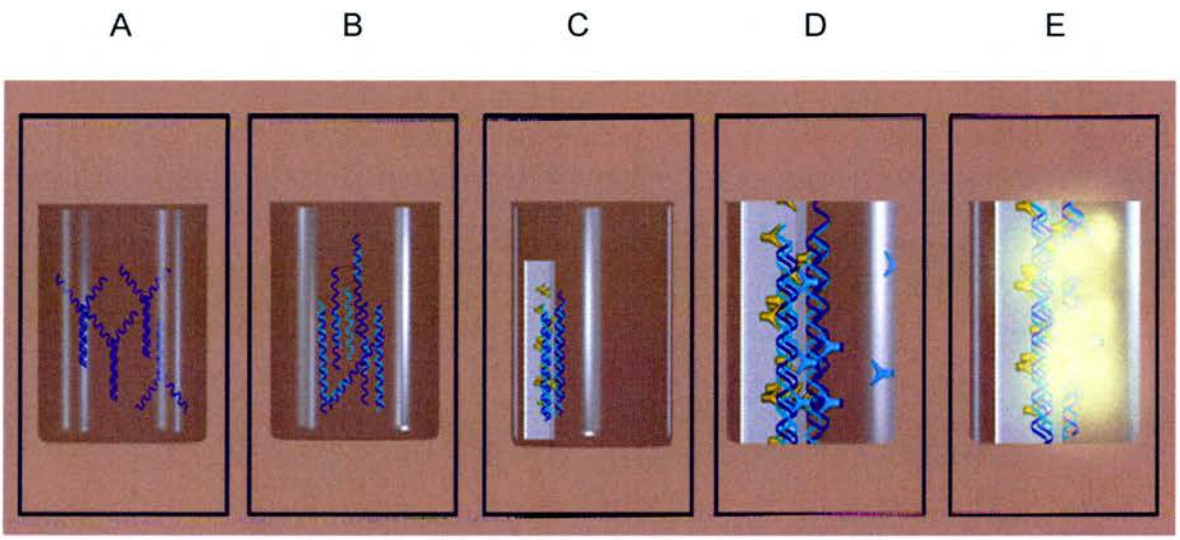
HC requires hybridisation of long single-stranded, or multiple oligonucleotide RNA probes to the whole denatured HPV genome in solution. The solution is added to a tube containing immobilised RNA-DNA antibodies conjugated to alkaline phosphatase on the tube wall. The RNA-DNA hybrids then bind in a non-sequence dependent fashion to the antibodies. The detection method uses a chemiluminescent substrate that attaches to the hybrid and provides a semi-quantitative result. By using RNA probes specific for either grouped high- or low-risk HPV types, both the phenotype and viral quantitation can be related to other investigational parameters such as HIV infection, immunosuppression, and partner number.(174) (See Figure 2.3)

HC, and specifically HC-2 produced by Digene Corp. (Gaithersburg, MD. USA) is the only FDA approved method for cervical HPV DNA testing. Anal HPV testing, or any form of HPV testing in men is not FDA approved and is not in routine clinical use.

The use of HPV typing is currently indicated in reflex testing of liquid cervical cytology reported as ASC-US using the 2001 Bethesda terminology.(175). In particular HPV testing using the second generation Digene HC 2 kit which

differentiates high-risk (Group B) from low-risk (Group A) HPV by the use of specific primer sequences has shown clinical efficacy in the triage of ASCUS cytology to routine care or colposcopic referral.(176) HC is also used in conjunction with cytology testing to screen women over the age of 30 years - if both tests are negative subsequent testing may be performed every 3 years instead of annually.

Figure 2.3: Hybrid capture 2 methodology



From left to right the diagram shows DNA (dark blue) being denatured (A), the addition of an RNA probe (light blue) that hybridizes with complementary sequences of DNA (B), capture of the DNA/RNA hybrids to a solid phase RNA/DNA antibodies (C), labeling of the hybrids (D), and finally production and detection of a chemiluminescent signal (E). Image adapted from Digene Corp.

2.9.5.5 POLYMERASE CHAIN REACTION

In this method, target DNA is selectively amplified by the action of DNA polymerase on specific primers that can then be detected by DB or STH or line blot as in the Roche linear array assay test. PCR can detect between 10 and 100 DNA molecules in a specimen and is the most sensitive method available.(177) By combining the sensitivity of PCR with the use of specific primer sequences, individual HPV types and type variants can be isolated from a variety of clinical specimens and correlated

with the disease process present. This technique has provided evidence for the etiologic role of HPV in the development of cervical/anal dysplasia and cancer. PCR testing for HPV is not used in clinical settings, although PCR may prove useful in a clinical setting similar to reflex HC testing above.(178) PCR testing shows extreme sensitivity, although there are concomitant concerns related to false-positive results due to possible specimen contamination. It is also unclear whether positive results found by PCR technology indicate current productive, latent or resolved infection, or all three.(174) (172) (179)

2.10 SUMMARY

The clinical manifestations of anogenital HPV infection have been apparent for thousands of years, while more recent methods such as cytology and colposcopy have been able to detect the presence of sub-clinical infection. Given the established association of high-risk HPV infection and anogenital squamous cell cancer, DNA testing technology may allow triage of those individuals who may be at highest risk of developing cancer. Furthermore, there is hope that with the development and reported efficacy of prophylactic HPV vaccines, there may be a decrease in anogenital squamous cancers in the future.

CHAPTER 3

ANAL SQUAMOUS INTRAEPITHELIAL LESIONS

3.1 INTRODUCTION

Anal squamous intraepithelial lesions (ASIL) are the putative precursor to anal squamous cell cancer (SCC). This chapter will discuss the classification, epidemiology, pathology, and clinical management of ASIL.

3.2 THE EMBRYOLOGY OF THE ANAL CANAL

The anal canal develops from the embryological cloacal membrane that is the site of endodermal and ectodermal fusion. As a result, the anal canal has a squamocolumnar epithelial junction where rectal columnar epithelium meets the squamous epithelium of the anal canal, and may normally display metaplastic change.(180) (See Figure 3.1) These embryological, anatomical and histological characteristics are shared with the cervical canal and extend to the cellular pathological response of both these tissues to infection with HPV.(181)

3.3 HPV ASSOCIATED PATHOLOGY OF THE ANAL CANAL

The anal canal is prone to infection with genitotropic HPV that may result in the development of ASIL.(182) (183) (184) Both high-risk and low-risk HPV's are associated with ASIL. Low-risk HPV (predominantly types 6 and 11) and high-risk HPV (predominantly types 16 and 18) may be found in association with low-grade squamous intraepithelial lesions (LSIL) including condylomata, whereas high-risk HPV such as 16 and 18 may be found associated with LSIL but are almost exclusively associated with the presence of high-grade squamous intraepithelial lesions (HSIL).(115) (58) (185)

Figure 3.1: Normal anal canal

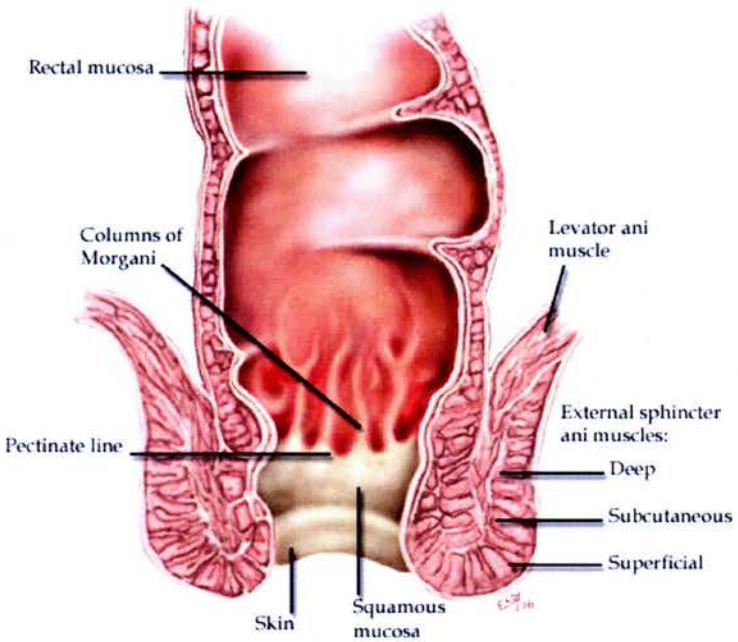


Image adapted from Ryan (186)

3.4 BETHESDA 2001 CYTOLOGY REPORTING SYSTEM

The Bethesda guidelines were originally developed for the classification of cervical squamous intraepithelial lesions (CSIL).(187) In this system, cytological evaluation of cellular material from the cervical canal, ideally including the squamocolumnar transition zone, and staining of the cells by the Papanicolaou method (188) is used to classify the degree of cervical cellular atypia. Exfoliative cytology and Papanicolaou staining has also been validated for use in the anal canal and is also reported using the 2001 Bethedsa system.(189) (175)

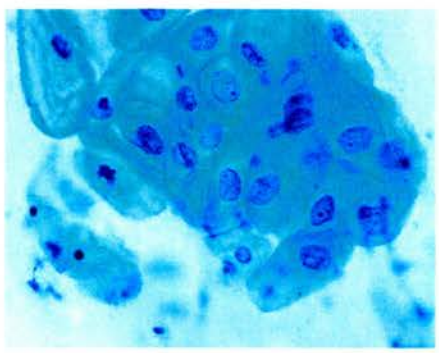
Table 3.1: The 2001 Bethesda system for reporting anal and cervical cytology (Abridged) (190)

SPECIMEN ADEQUACY Satisfactory for evaluation (*note presence/absence of endocervical/transformation zone component*) unsatisfactory for evaluation . . . (*specify reason*) specimen rejected/not processed (*specify reason*) specimen processed and examined, but unsatisfactory for evaluation of epithelial abnormality because of (*specify reason*)
GENERAL CATEGORIZATION (Optional) Negative for intraepithelial lesion or malignancy Epithelial cell abnormality Other
INTERPRETATION/RESULT **Negative for Intraepithelial Lesion or Malignancy** Organisms *Trichomonas vaginalis* Fungal organisms morphologically consistent with *Candida* species Shift in flora suggestive of bacterial vaginosis Bacteria morphologically consistent with *Actinomyces* species Cellular changes consistent with herpes simplex virus Other non-neoplastic findings (*Optional to report; list not comprehensive*) Reactive cellular changes associated with inflammation (includes typical repair) radiation intrauterine contraceptive device Glandular cells status posthysterectomy Atrophy **Epithelial Cell Abnormalities** Squamous cell Atypical squamous cells (ASC) of undetermined significance (ASC-US) cannot exclude HSIL (ASC-H) Low-grade squamous intraepithelial lesion (LSIL) encompassing: human papillomavirus/mild dysplasia/cervical intraepithelial neoplasia (CIN 1) High-grade squamous intraepithelial lesion (HSIL) encompassing: moderate and severe dysplasia, carcinoma in situ (CIN 2 and CIN 3) Squamous cell carcinoma Glandular cell Atypical glandular cells (AGC) (*specify endocervical, endometrial, or not otherwise specified)* Atypical glandular cells, favor neoplastic (*specify endocervical or not otherwise specified*) Endocervical adenocarcinoma in situ (AIS) Adenocarcinoma **Other** (*List not comprehensive*) Endometrial cells in a woman over 40 years of age

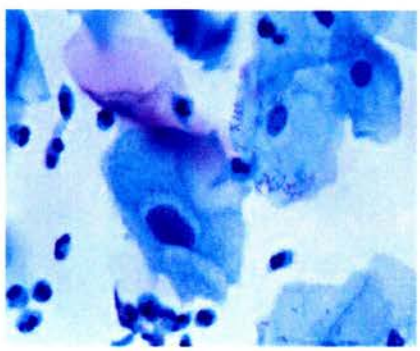
3.4.1 THE CYTOPATHOLOGICAL CLASSIFICATION OF SQUAMOUS INTRAEPITHELIAL LESIONS

Increasing degrees of squamous cytological abnormality are shown in Figure 3.2.

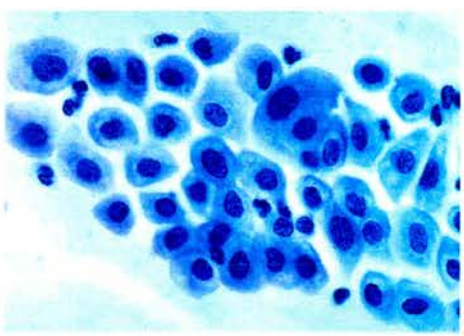
Figure 3.2 Normal, ASCUS, ASCH, LSIL, HSIL, and squamous cancer cytology



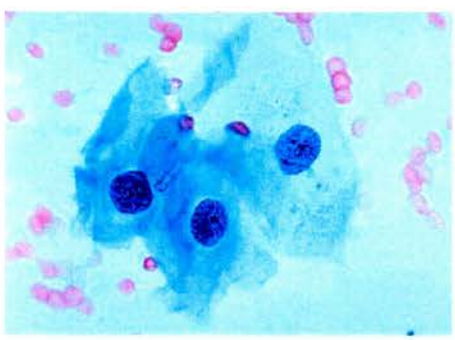
Normal



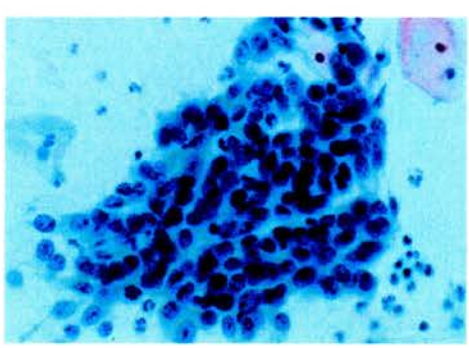
ASCUS



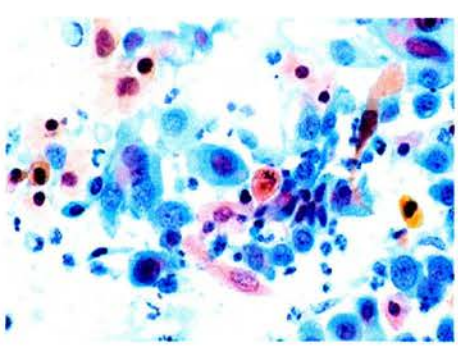
ASCH



LSIL



HSIL



Squamous cancer

Images reproduced with permission of the National Cancer Institute

3.4.2 ANAL AND CERVICAL HISTOPATHOLOGY

Cervical and anal dysplasia are defined by the presence of abnormal cells with an immature, basaloid appearance present in varying degrees of epithelial thickness from the basement membrane towards the epithelial surface. These cells characteristically exhibit an increased nuclear to cytoplasmic ratio, dark staining/polychromatic chromatin, and may undergo mitosis in cell layers normally characterised by more differentiated cells.

Low-grade dysplasia is diagnosed when abnormal cells replace the lower third of the epithelium and is reported histologically as cervical intraepithelial neoplasia grade 1 (CIN 1) and anal intraepithelial neoplasia grade 1 (AIN 1). The cellular changes in low-grade dysplasia include the presence of koilocytes, enlarged cells with a halo of clear cytoplasm surrounding the nucleus that is associated with HPV infection.

High-grade dysplasia encompasses moderate and severe dysplasia and is defined histopathologically as abnormal basaloid cells comprising up to two thirds (CIN 2/AIN 2) and between two thirds and the full thickness (CIN 3/AIN 3) of the epithelium. CIN 3 and AIN 3 were previously referred to as carcinoma in situ (CIS).(187)

High-grade CSIL, is now generally accepted to be the precursor lesion of cervical cancer (11) and is causally related to infection with oncogenic types of HPV.(191) Likewise it is thought considered that high-grade ASIL is the precursor of anal cancer. Figure 3.3 shows a diagrammatic representation of anal and cervical dysplasia, and Figure 3.4 shows increasing degrees of anal dysplastic change in anal biopsy specimens.

Figure 3.3: Diagrammatic representation of dysplasia

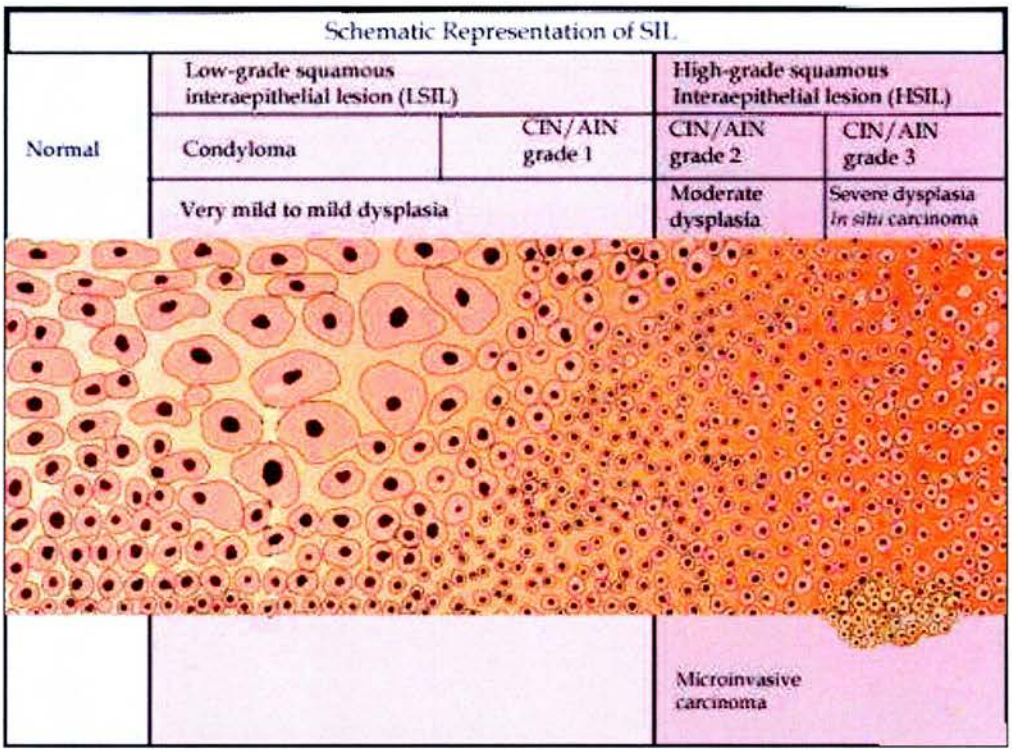
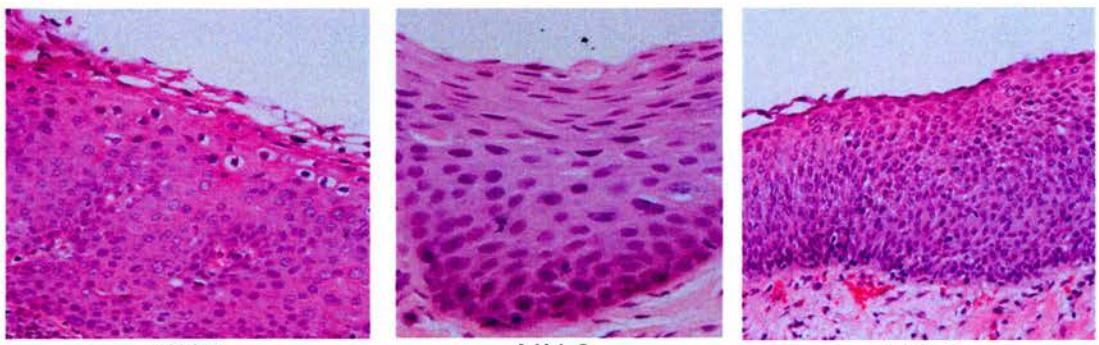


Image adapted from Palefsky (192)

3.5 CLINICAL ASSOCIATIONS OF ASIL

Development of anal canal SCC is associated with both anal canal HPV infection (193, 194) (185) (103) (195) and ASIL.(196) (197) (198) (91) Early SCC may present with symptoms of anal pain, bleeding, and sometimes the presence of a palpable mass lesion. These symptoms are common in the general population and may occur with herpes simplex virus (HSV) infection, anal warts, anal fissure, and hemorrhoids – and so may be ignored by the individual. This may particularly be the case in MSM who commonly have anal symptoms related to the practice of receptive anal intercourse.(199)

Figure 3.4: Anal intraepithelial neoplasia grade 1, 2 and 3



Images courtesy of Dr Galen Cortina, Department of Pathology, UCLA

3.6 EVIDENCE FOR ASIL AS A PRECURSOR OF ANAL SQUAMOUS CELL CANCER

The progression of high-grade anal dysplasia to anal cancer has not been definitively proven. However, considering the cervical paradigm of progression from high-grade cervical dysplasia to cervical cancer, in addition to the associations below, it is biologically plausible that this is also the case for high-grade anal dysplasia. The observed parallels between anal and cervical epithelial dysplasia include:

- ASIL and anal cancer are both associated with HPV, and similar HPV risk-types as CSIL and cervical cancer. (185) (57, 200) (201) (202) (192) (203) (204) (205)
- CSIL/ASIL and cervical/anal cancer are similar pathologically.(114)
- In populations known to be at risk of anal cancer there is a high incidence of ASIL.(206) (59) (207)
- Cervical/anal HSIL is frequently found overlying cervical/anal cancer.(57, 200)

- There is documented evidence of progression from high-grade anal dysplasia to anal cancer in both iatrogenic and HIV-induced immunosuppression.(208) (209)
- There is documented progression of Bowen's disease (perianal AIN-3) to cancer.(210)
- Chromosomal abnormalities found in high-grade anal dysplasia are also found in cervical cancer.(181)

Thus, analogous to treatment of cervical HSIL to prevent cervical cancer, ablation of anal HSIL may be effective in reducing the incidence of anal cancer.

3.7 SCREENING FOR ASIL

The institution of routine cervical cytology screening has contributed to the dramatic decrease in the incidence of cervical cancer in the US.(211) The similar etiology and histopathology of ASIL and CSIL has led investigators to translate the screening methods used for the cervix to the anal canal, and to assess the technique of anal cytology as a screening tool. Palefsky *et al* reported the sensitivity of anal cytology to detect biopsy-proven anal dysplasia in HIV-positive men to be equal to that of cervical cytology for the detection of cervical dysplasia when ASCUS was included in the 'abnormal' category.(196) The sensitivity of this technique was lower in HIV-negative men (50 percent) compared with HIV-positive men (81 percent), possibly reflecting the presence of less widespread disease than is seen in an HIV-positive population.(196) As in the cervix, histopathology is considered necessary to grade the severity of the disease as this cannot be accurately established by cytology alone.(196) Collection of specimens for histopathologic evaluation is accomplished

through visualisation and sampling of lesions identified by high-resolution anoscopy (HRA).

3.7.1 CERVICAL VERSUS ANAL CYTOLOGY SCREENING

There are important technical considerations that differ between anal and cervical cytology. Cervical cytology using a spatula and endocervical brush can typically yield more than 50,000 epithelial cells per sample (212) which is significantly more than most anal samples.(213) This, combined with possible cellular artifact caused by air drying prior to ethanol fixation are two of the main causes of anal samples being inadequate for cytopathological interpretation.(213) Other factors that may increase the difficulty of interpretation of anal cytology samples are faecal and bacterial contamination.(189)

There are two available methods for anal cytology sample collection. The first and most established is that of alcohol fixation of the cytology sample and subsequent staining by the Papanicolaou method.(196) The other more recent method uses cells that are collected then released into a methanol containing fixative before being processed and subsequently stained by the Papaniculaou method.(214) This liquid cytology method has been shown to have greater diagnostic accuracy when used for cervical cytology.(215) (212) (216) While liquid cytology reduces faecal and bacterial contamination for anal samples, a comparative study showed no difference between the two collection methods.(189) However, a potential research advantage of liquid cytology is that the fluid may also be tested for HPV typing.(176)

As with cervical cytology, the experience of the clinician taking an adequate cytology specimen and the pathologist's experience in interpreting the anal specimen is essential to the diagnostic process.(217) Inter-observer differences in diagnosis also

may occur with interpretation of histopathology samples. In one study by Colquhoun *et al* there was only moderate agreement between three pathologists assessing 190 anal biopsy specimens.(218)

3.7.2 ASIL SCREENING GUIDELINES

Chin-Hong and Northfelt have published guidelines on screening for individuals at risk of developing anal cancer.(114) (219) (See Table 3.2)

Table 3.2 High-risk groups for anal cancer

HIV negative men with a history of receptive anal intercourse (RAI)
HIV-positive men regardless of CD4 level
HIV-negative women with high-grade CSIL or high-grade vulval SIL
HIV-positive women regardless of CD4 level
Solid organ transplant recipients
Individuals with perianal condylomata accuminata

The goal of screening for ASIL is ultimately to identify anal HSIL. An anal screening program based on the cervical screening paradigm would rely on exfoliative cytology as the initial screening test. The goal of screening at this stage would be to identify individuals who need to undergo HRA. In this respect, anal cytology should be classified as "normal" versus "abnormal", i.e. anyone with ASCUS, ASCH, LSIL or HSIL should undergo anoscopy. It would also be inappropriate to depend on anal

cytology grade as this is poorly predictive of anal histopathological diagnosis on biopsy following HRA.(220)

3.7.3 COST EFFECTIVENESS OF SCREENING FOR ASIL

There have been two key papers published by the same group examining the cost-effectiveness (CE) of anal cytology in screening HIV-positive and HIV-negative MSM.

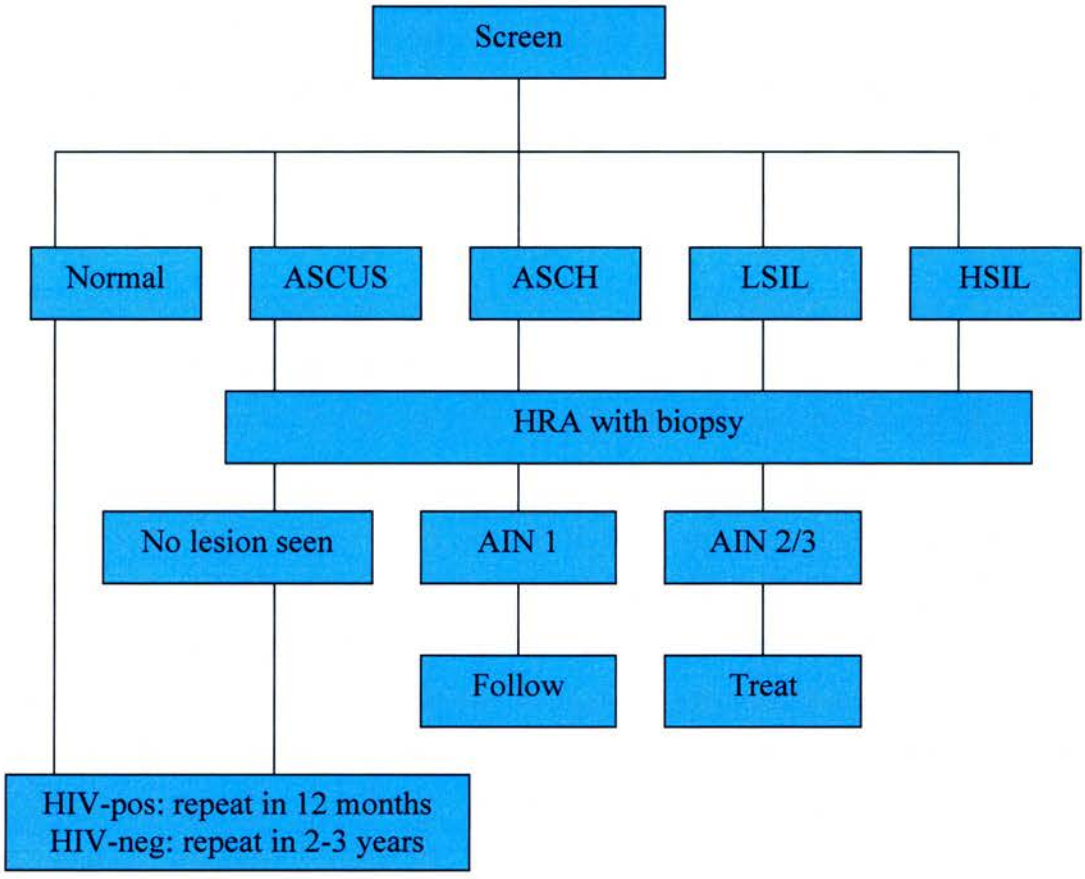
Goldie *et al* reported the CE of performing anal cytology screening in a male homosexual and bisexual HIV-positive population. A state-transition Markov model was developed to calculate lifetime costs, life expectancy, and quality adjusted life expectancy for no screening versus several screening strategies. Strategic variables included screening interval, stage of HIV infection, and progression or regression of anal lesions. The results of this study showed that screening every 2 years beginning in the early stages of HIV infection was associated with an incremental cost-effectiveness ratio of approximately $13,000 per quality-adjusted life year (QALY) saved. This compares well with the accepted use of trimethoprim-sulphamethoxazole for *Pneumocystis carinii* pneumonia prophylaxis in HIV disease ($13,000/QALY) and is considerably less expensive than triennial cervical cancer screening in HIV-negative women ($180,000/QALY). Annual anal cytology screening was also cost-effective and was recommended by the authors as the screening interval of choice for HIV-positive MSM.(221)

A subsequent study by the same group again using a state-transition Markov model showed that screening HIV-negative homosexual and bisexual men with anal cytology every 2-3 years provided life expectancy benefits comparable with accepted preventative health measures, and would be cost-effective.(222)

3.7.4 SCREENING FOR ASIL IN PRIMARY CARE.

Current clinical practice at UCSF is shown in Figure 3.5.

Figure 3.5: Current suggested anal cytology screening practice



Adapted from Chin-Hong (114)

For negative cytology reports, as with cervical cytology, the sample should be repeated due to the possibility of false-negative reporting.(196) The cytology sample may be repeated on an annual or biannual basis depending on HIV status.

3.8 OBTAINING AN ANAL CYTOLOGY SAMPLE

To obtain a sample for anal cytology, subjects should be advised to refrain from placing anything in the anal canal for 24 hours before the anal cytology swab is taken. This includes using an enema or douche, or having receptive anal sex. These measures aim to increase the yield of exfoliated anal canal cells when the cytology swab is taken. With the patient in the left lateral position, a tap water or 0.9% saline-moistened Dacron™ swab (Baxter Healthcare Corporation, McGraw Park, Illinois, USA) is gently inserted at least 2 inches beyond the anal margin, so as to be proximal to the anorectal transition zone. Cotton swabs should not be used as it is more difficult to release anal cells onto the glass slide although nylon brush endocervical swabs have been used in some centres.(223) With firm lateral pressure and a spiral motion to sample the entire anal circumference, the swab is withdrawn over 10 seconds. The swab can either be smeared onto a glass slide and the slide immersed immediately in ethanol fixative, or agitated vigorously in ThinPrep™ liquid collection media to disgorge cellular material.(189) Regardless of collection method, anal cytology grading follows the standard 2001 Bethesda system as above.(224)

3.9 HIGH-RESOLUTION ANOSCOPY EVALUATION OF THE ANAL CANAL

Following a diagnosis of abnormal anal cytology, it is recommended that patients undergo high-resolution anoscopy (HRA). The purpose of HRA is to identify the source of the dysplastic cells and perform a biopsy to establish the histological grade of the lesion(s).

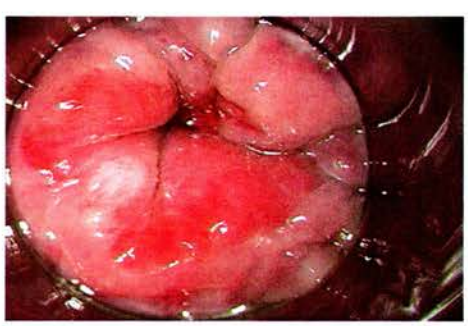
With the patient in the left lateral position a visual inspection of the peri-anal area is made, followed by a digital examination to define masses or tender areas. Lesions that

are firm, immobile, and painful should raise suspicions of invasive cancer. A clear plastic anoscope is lubricated and inserted into the anal canal. For patients with anal discomfort the anoscope may be lubricated with xylocaine jelly. A gauze swab soaked in 3 percent acetic acid and wrapped around a Q-tip is inserted through the anoscope. Following this, the anoscope is withdrawn with the swab left inside in direct contact with the anal mucosa for 1 minute before removal.(196) Abnormal squamous epithelium will turn white in the presence of the acetic acid (hence the term 'aceto-whitening'), although the change is transient requiring re-application of acetic acid. Lugol's iodine solution may also be used to confirm suspicion of high-grade dysplasia. Typically HSIL does not take up the iodine stain and remains yellow, while normal tissue turns a deep brown colour that relates to its glycogen content. This test has not been verified for the anal canal but is used in assessing dysplasia in both the cervix and oesophagus.(225) (226) (227) The colposcopic features of a high-grade lesion in the anus correspond to those of high-grade lesions in the cervix, and thus the established colposcopic criteria for identifying cervical HSIL may serve as a guide for the clinician performing anal biopsies.(160) (See Figure 3.6) Intra-anal biopsies may be performed with standard endoscopy forceps with a 2.3 mm non-serrated cup without local anesthetic, the patient usually only being aware of a tugging sensation. Hemostasis can then be achieved with ferrous subsulphate (Monsell's) solution or local pressure applied with a cotton-tipped swab under direct visualisation. Following any anal biopsy, the individual should be counseled with regard to the rare complications of bleeding and infection. Subjects with a low granulocyte or platelet count should not undergo anal biopsy, and patients taking acetylsalicylic acid (ASA) or other platelet inhibitors should defer the biopsy until they have stopped therapy long enough for the anti-platelet activity to abate. Standard antibiotic prophylaxis

guidelines should be followed in subjects with prosthetic or abnormal heart valves. Lesions overlying hemorrhoids are prone to significant bleeding and it is more appropriate that anal surgeons, who can control haemostasis more aggressively, perform biopsies of those lesion(s) in patients who cannot discontinue anticoagulants.(206)

The external anal margin and peri-anal skin should also be carefully examined for abnormality after the application of 3 percent acetic acid, however abnormal changes in this site are less well characterised. When external biopsies are indicated, local anesthesia with lignocaine is required before biopsy with baby Tischler forceps. The subject should be counseled regarding local hygiene measures and the use of simple analgesia if required.

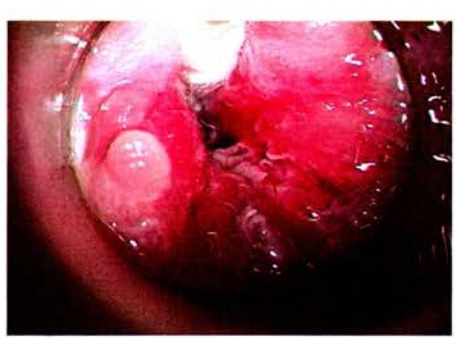
Figure 3.6 High-resolution anoscopic appearances of normal anorectal transition zone, condylomata/LSIL, HSIL, and squamous anal cancer



Normal anorectal transition zone

Condylomata/LSIL

HSIL



Squamous anal cancer

Images courtesy of the UCSF Anal Neoplasia Study

3.10 EVALUATION OF HRA

There are few published reports of the visual correlation of anal epithelium and pathological grade. The first was published by Scholefield *et al* and showed a high correlation for normal and high-grade changes, but was less accurate for low-grade dysplasia and non-condylomatous HPV infection.(228) More recently Matthews *et al* showed a sensitivity of 59% and specificity of 75% between visual criteria and anal histopathological diagnoses using the categories of invasive carcinoma, HSIL, LSIL, and other, with visual assessment of HSIL showing a positive predictive value of 74.7%.(229)

3.11 RISK FACTORS FOR ASIL

Risk factors for the development of ASIL in men are summarised in Table 3.3.

Table 3.3: Risk-factors for ASIL in men

HPV infection (197) (91) (206) (230)
HIV infection especially with a lower CD4 count (198) (230) (62) (91) (86) (109) (58, 59, 231)
Receptive anal intercourse (106)
History of rectal discharge (91)
History of genital warts (91)
History of injection drug use (230)
Current cigarette smoking (230) (109)

The level of HPV in the tissue may also be important in the development of ASIL (198) as a high level of HPV was significantly associated with ASIL in both HIV-positive and HIV-negative men in one large study.(91) Additional risk factors for ASIL in women include concomitant high-grade cervical or vulval lesions,(232) (209) cervical cancer (233) (234), or vulval cancer (235). Iatrogenic immunosuppression such as that required for solid organ transplantation has also been reported to increase the prevalence of HPV-related anal disease.(235)

3.12 NATURAL HISTORY OF ASIL

Studies are ongoing to characterise the natural history of HPV related ASIL in both HIV-negative and HIV-positive populations. To date, risk-factors for progression from normal anal epithelium or low-grade dysplasia to high-grade dysplasia include immunosuppression by HIV,(206) (102) detection of multiple HPV types,(206) history of genital warts,(91) and history of rectal discharge.(91) Furthermore, several authors have reported other factors that may indicate or increase the risk of progression to cancer in susceptible individuals. Ogunbiyi *at al* suggested that expression of the oncogene *c-myc*, and subsequent detection of *c-myc* protein may be linked to progression of high-grade dysplasia as may the increasing presence of nucleolar organiser region associated proteins.(236, 237) Xi *et al* reported that infection with non-prototype (NPL) HPV 16 was a risk factor for the development of carcinoma in situ (CIS) in MSM compared with prototype like (PL) HPV 16.(238) This finding was supported by Da Costa *at al* who reported an increased risk of anal HSIL with NPL HPV 16 after controlling for HIV infection.(53) Haga *et al* examined the genetic changes in increasingly dysplastic anal epithelium by comparative genomic hybridisation in HIV-positive and HIV-negative MSM. There was a

significantly increased genetic change with increasing grades of anal dysplasia, most commonly mapped to chromosome arm 3q. This finding is analogous to genetic changes demonstrated in cervical cancers, suggesting a common pathway for the two HPV associated squamous neoplasias.(181) (239)

Compared to the cervical literature, anal dysplasia as a clinical entity has only relatively recently been the focus of research attention and there is only limited information from the small number of natural history studies performed. Consequently there has been little direct evidence documenting progression of high-grade anal dysplasia to anal cancer that would help support the implementation of an anal cytology screening program. However, a recent prospective observational cohort study of HIV-positive MSM with biopsy proven high-grade anal dysplasia reported that anal cancer developed in 3 of 40 men during follow-up.(208). In another cohort study of individuals with high-grade anal dysplasia and median follow-up of 63 months, 3 of 6 individuals with non-HIV induced immunosuppression developed anal cancer. There was no progression in the 29 immunocompetent individuals, 28 of whom had received surgical treatment for high-grade dysplasia.(209)

3.13 DEMOGRAPHICS OF ASIL

No comprehensive information is currently available on the demographics of ASIL in the general population, as centres investigating this condition have focused primarily on populations of MSM. The MSM studied tend to be in men between the ages of 30 and 50, are living in American cities, and have a high prevalence of HIV infection.

The available literature reports that abnormal anal cytology is present more frequently in HIV-positive MSM. Rates of any anal cytological abnormality range from 28 to 56 percent in HIV-positive MSM and 4.7 to 8 percent in HIV-negative MSM.(91) (109)

(240) (241) (242) (230) In addition, natural history studies confirm an increased prevalence of ASIL over time in HIV-positive MSM. In one study of MSM, 27 percent of subjects were diagnosed with ASIL at baseline and this figure increased to 65 percent after an average of 17 months.(242)

A recent study in HIV-negative North American MSM reported that the prevalence of anal cytological abnormalities was constant in this population over a wide age range.(243) Specifically the prevalence of LSIL was 15% and associated with reports of more than five male receptive anal sex partners, any use of alkyl nitrites in the previous 6 months, use of injection drugs two or more times per month during the previous 6 months, older age at first receptive anal intercourse, and infection with a greater number of HPV types.(243) The prevalence of HSIL was 5 % in this population and associated with any HPV infection and infection with multiple HPV types.(243)

ASIL is also more prevalent in HIV-positive women with prevalence ranging from 15 to 42 percent compared with HIV-negative women who have a reported prevalence of 1.4 to 12 percent.(62) (198) (244) (245) (116)

3.14 CONDYLOMATOUS LESIONS AND HIV INFECTION

There are differences in the clinical presentation of ASIL in an HIV-positive population, compared to an HIV-negative population. Exophytic genital warts are usually associated with infection with low-risk HPV (types 6 and 11) and a LSIL cellular change. However, in an HIV positive population, high-risk HPV and associated high-grade dysplastic change has been isolated from exophytic lesions more frequently than in HIV-negative controls. This suggests that biopsy to define histological grade of dysplasia should be considered in an HIV-positive population

before standard destructive therapy of these lesions to exclude early HSIL or early SCC.(246) (247) Additionally, at HRA the appearance of internal anal lesions (flat or exophytic) have not been shown to correlate with detected HPV risk-type.(248)

3.14.1 HIV/HPV INTERACTIONS

As previously discussed, HIV/HPV interactions in the context of impaired cell mediated immunity (CMI), altered mucosal cytokine patterns and the interaction of HIV *tat* protein in increasing E6 and E7 HPV expression may explain the increased incidence and progression of LSIL to HSIL in HIV-positive MSM.(115) There is inadequate available data addressing this issue in women although it may be reasonable to assume a similar mechanism.

3.14.2 ANAL DYSPLASIA AND HIGHLY ACTIVE ANTIRETROVIRAL THERAPY

Recent advances in the management of patients with HIV infection, including the use of potent combination antiretroviral therapy have been associated with decreased mortality and morbidity in these individuals. As a consequence, HIV-positive individuals are exposed for a greater period of time to high-risk HPV infection,(196) (88) (110) high level HPV infection,(249) infection with multiple HPV types,(241) and are experiencing a high prevalence of HSIL.(230) (250) Highly active antiretroviral therapy (HAART) is associated with improvements in functional cell mediated immunity as measured by increasing CD4 lymphocyte counts, improved response to recall antigens, and decreased incidence of opportunistic infections. These immunological changes may be postulated to have a beneficial effect on HPV infection and so reduce the prevalence of anal dysplasia. The effect of HAART on anal dysplasia has recently been investigated in a cohort of HIV-positive MSM. Anal

cytology, anal biopsy, HPV typing and level of infection were collected at least six months before, and at least six months after starting HAART. This study showed that HPV isolation, level of HPV, and grade of ASIL was similar between the two investigational points.(251)

Furthermore HAART therapy was associated with the presence of anal dysplasia in multivariate analysis of a cohort of HIV-positive MSM in San Francisco, in addition to detection of multiple HPV types.(252) Further investigation of variables such as HAART efficacy, baseline CD4 counts and extended length of HAART treatment (including HAART failure) are now necessary to more fully define the response of anal HPV and abnormal anal cytology to combination antiretroviral therapy.

3.15 TREATMENT OF ASIL

Therapy for anal dysplasia is usually only considered for those individuals with AIN 2/3 since these lesions are thought more likely to progress to anal SCC. However, many patients opt for therapy for a variety of other reasons including symptoms (burning, itching, and bleeding) and psychological discomfort. Untreated patients with LSIL, especially if HIV-positive, are followed approximately every six months because of the high rate of progression to HSIL.(206) However, individuals with AIN 1 who desire therapy for treatment of symptoms or psychological discomfort should also be considered for therapy. (See Chapter 2)

Experience is limited with current modalities of treatment. To date there has only been one formal efficacy trial reported in the literature containing efficacy data for treatment of AIN 2/3 (see below).

3.15.1 TREATMENT OF LOW-GRADE ANAL DYSPLASIA/ CONDYLOMATA

This topic is covered in Chapter 2. It is noteworthy that, in the context of HIV infection, there is an increased risk of clinical recurrence of condylomata when compared to an HIV-negative population.(88)

3.15.2 TREATMENT OF HIGH-GRADE ANAL DYSPLASIA

3.15.2.1 COLD SCALPEL EXCISION/ELECTROFULGURATION

Cold-scalpel excision of dysplastic tissue under anesthesia has, until recently, been the mainstay of therapy. This method is best utilized when there is discrete, limited, non-circumferential disease. Extensive disease may require multiple staged operations to clear the lesions and prevent anal stenosis. Although usual anaesthetic and surgical complications such as haemorrhage and infection may occur, they are rare with this procedure. Post-operative pain however has proved difficult to control and may be significant for up to 2 weeks post-surgery, particularly with bowel movements. In addition to cold-scalpel resection, electrofulguration may be carried out concomitantly with similar side effects and complications.

Until recently there has been little information on the follow-up and efficacy of these treatments for anal disease. A group working at UCSF published data on the efficacy of excision/cauterization of anal HSIL identified by HRA in 37 HIV-positive and 8 HIV-negative MSM. Results showed that although this procedure was safe, there was a persistence/recurrence in 79 percent of HIV-positive patients (23/29) treated with a mean follow-up of 28.6 +/- 12.9 months. However, no HIV-negative patients (0/8) developed recurrent HSIL during follow-up of 32.3 +/- 20.6 months. The authors suggested multiple staged procedures may be appropriate for HIV-positive patients.(253)

3.15.2.2 LASER ABLATION/PHOTODYNAMIC THERAPY

Laser ablation of dysplastic tissue may be undertaken in the operating room, but experience with this relatively new technique for this indication is limited, as is the case with photodynamic therapy.(254)

3.15.2.3 INFRARED COAGULATION

The mechanism of action of the IRC is described in Chapter 2. The IRC (See Figure 3.7) has recently been used as an alternative to surgical excision in individuals with HSIL. A retrospective review was undertaken in 68 HIV-positive MSM with biopsy proven high-grade anal dysplasia that underwent IRC treatment. 165 lesions in 68 patients were treated and at follow-up only 46 lesions persisted. Subsequently 44 patients developed new or persistent high-grade lesions within a median time of 217 days. When these patients were re-treated the incidence of new or persistent high-grade lesions fell from 58% to 40% respectively. No serious adverse events were reported following the procedure and no patients developed anal cancer during follow-up.(136)

Figure 3.7 The Infrared coagulator

IRC2100TM, Redfield Corp., Rochelle Park, NJ (with permission)

3.16 EXTERNAL ASIL

Perianal HSIL has proven malignant potential.(255) In 1912 Bowen was the first to describe the perianal lesions with intraepithelial keratinocytic atypia that bear his name.(256) The lesions typically present as chronic, red or hyperpigmented, well-defined scaly plaques. Histological examination shows AIN 3 but no invasion of the dermis. The exact lifetime risk of progression from Bowen's disease to invasive SCC is unknown but may be up to 6%.(257)

3.16.1 TREATMENT OF EXTERNAL ASIL

Treatment modalities for Bowen's disease include electrodessication and curettage and excisional surgery with subsequent follow-up.(258) (259) More recently there have been case reports of Bowen's disease responding to topical Imiquimod and 5-fluorouracil.(260) (261) Other treatment modalities include the use of used include cryotherapy,(262) laser ablation,(263) and photodynamic therapy.(264)

3.17 SUMMARY

The anal and cervical canal share many common features. They are both derived from embryologically similar tissue, have a squamocolumnar epithelial transition zone, and support anogenital HPV infection that may cause dysplastic change and lead to squamous malignancy. For more than 50 years screening cervical cytology has been performed in developed countries to detect cytological abnormalities that may progress to cervical cancer. More recently cytological testing has been validated for the anal canal to detect anal squamous intraepithelial lesions. Anal cytological abnormalities may be defined histopathologically following high-resolution anoscopy and anal biopsy, analogous to cervical colposcopy, with subsequent treatment of high-

grade dysplastic lesions. It is yet unproven, although biologically plausible, that high-grade anal lesions progress to anal cancer and that by treating high-grade lesions this progression may be abrogated.

CHAPTER 4

ANAL SQUAMOUS CELL CANCER DEVELOPING IN THE CONTEXT OF A PREVIOUS HIGH-GRADE ANAL DYSPLASIA DIAGNOSIS

4.1 INTRODUCTION

Anal and cervical squamous intraepithelial lesions and cancer share many common features as described in Chapter 3. In this chapter anal squamous cell cancer will be considered in the context of anal dysplasia progression, and the demographics of individuals who progressed from documented high-grade anal dysplasia to anal cancer.

4.2 THE ANAL AND CERVICAL CANAL

Detection of CSIL by cervical cytology screening programs, followed by ablative treatment of the lesions is considered to have contributed substantially to the decline in incidence of cervical cancer in the United States from 35 per 100 000 to 8 per 100 000 since the introduction of this program over 50 years ago.(211) However, concurrent with this drop in the incidence of cervical cancer, there has been a rise in recent years in the incidence of anal cancer in both men and women.(265) (58) (12)

4.3 ANAL SQUAMOUS CELL CANCER (SCC)

4.3.1 ANAL SCC INCIDENCE

In the United States, the current incidence of anal squamous cell cancer (SCC) in the general population is approximately 1.4 per 100,000.(266) However, there is evidence of increased risk of anal cancer certain groups, specifically MSM. (267) (268) (269) (270) (271) Considering a population of never married men, a surrogate for MSM, prior to the clinical manifestations of the HIV epidemic, the rate of anal cancer was estimated to be as high as 35 per 100,000.(272) Moreover, recent data indicates that the incidence of anal cancer in HIV-positive MSM is approximately double that of HIV-negative MSM, with Frisch *et al* reporting that the relative risk of anal cancer

among HIV-positive men was 37 times higher than the general population.(273) (274, 275) (276) (277, 278) In San Francisco County, the incidence of anal cancer among all men aged 40-64 years more than quadrupled from a pre-HIV epidemic period (1973-1978) to during the HIV epidemic period (1996-1999).(279) The incidence of anal cancer in both HIV-negative and HIV-positive MSM is therefore as high, or higher than the current incidence of cervical cancer in the general female population and is similar to the incidence of cervical cancer prior to the introduction of cervical cytology screening.(115) Other groups at increased risk of anal cancer include women with concurrent high-grade cervical disease or cervical cancer,(232) (228) high-grade vulval dysplasia or vulval cancer,(280) and anal warts.(193) Recently HIV-positive women were shown to be at increased risk of anal cancer compared to women in the general population with a relative risk of 6.8.(198, 273) (62) The rate of anal cancer is also increasing in the context of iatrogenic immunosuppression such as occurs with solid organ transplantation (89, 281) and with additional significant morbidity from other non-anogenital cutaneous and mucosal HPVs.(282)

4.3.2 ANAL SCC TUMOUR SITE

In 1987 the World Health Organization defined anal canal tumours as arising from the area between the dentate line and the anal verge, with anal margin tumours defined as arising distal to the anal verge.(283) There are however various definitions of the anal canal:

- **Classic anatomic definition of anal canal:** between proximal and distal margins of internal sphincter muscle, which includes part of rectum.
- **American Joint Committee on Cancer definition of anal canal:** begins where the rectum enters the puborectalis sling at the apex of the anal sphincter

complex (palpable as anorectal ring, but difficult for pathologists to identify), and ends at the squamous mucocutaneous junction with perianal skin; includes 1-2 cm of rectal-type glandular mucosa and possibly transitional mucosa at the dentate line.

- **Histologic definition of anal canal:** anal transitional zone and squamous epithelium to the perianal skin.

4.3.3 ANAL SCC EPIDEMIOLOGY

Anal cancer is a rare tumour in the general population, accounting for 1-2% of all large gastrointestinal malignancies in the US, and accounts for just 5% of anorectal malignancies. (186) (284) (285) (286) The various histological types include squamous cell (80% of tumours) and its variants (cloacogenic, basaloid and transitional tumours) in addition to adenocarcinoma (5-10%),(287) melanoma, leiomyosarcoma and carcinoid tumours.(288)

The increasing incidence of SCC (265) (270) has been related to the HIV pandemic (265) and changing patterns of sexual behaviour, particularly the trend for increasing numbers of sexual partners.(270)

4.3.4 ANAL SCC DEMOGRAPHICS

The Surveillance, Epidemiology, and End Results (SEER) program, a system of population-based US tumour registries reported an increase in incidence of predominantly squamous cell anal cancer in the general US population between the years 1973-1979 and 1994-2000.(266) While there was previously a female predominance in anal cancer diagnoses there is now sex equality with the largest notable increase in incidence in Black males. In numerical terms, individuals with an

anal cancer diagnosis are most commonly Caucasian and present in the sixth and seventh decades of life.(286) (289) (290) (291) (292) (266)

4.3.5 ANAL SCC RISK-FACTORS

Published risk factors for anal cancer include immunosuppression (HIV, solid organ transplant recipients, systemic corticosteroids), high-risk HPV infection, higher numbers of sexual partners, cervical cancer in women, chronic anal fistula or fissure, previous radiation, Crohn's disease, and cigarette smoking.(288) (186, 193) (209)

4.3.6 ANAL SCC CLINICAL PRESENTATION

Anal cancer may present with a palpable mass (100%), bleeding (78%), pain (70%), change in bowel habits (29%), discharge (20%), and pruritus ani (20%).(288) (293) However, no symptom is pathognomonic. Other anal conditions such as hemorrhoids, fissures, leucoplakia and fistulas may be present concomitantly and share similar symptomatology.

At presentation the majority of tumours have infiltrated through sphincteric muscle into pelvic soft tissue,(294) (295) and 10-50% of patients have regional lymph node spread,(294) (296) (297) (285) while 5-10% of patients have distant metastases.(298)

4.3.7 ANAL SCC TREATMENT

Previously, anal cancer was treated with radical surgery, often requiring an abdomino-peroneal resection necessitating the fashioning of a colostomy. More recently, similar treatment efficacy has been achieved by using combined chemotherapy and radiotherapy, with the additional benefit of preserving anal sphincter function (85%

versus 9%).(299) (300) (301) (302) (303) Although therapeutic regimes vary, the elements are similar and include an infusion of 5-fluorouracil, a bolus of mitomycin C, followed by megavoltage external beam radiotherapy.(288)

The mitomycin C component has been controversial due to additional hematological toxicity and questionable efficacy particularly with larger tumours, but shows improved responses in combination with radiotherapy and chemotherapy in smaller tumours.(304) More recently cis-platinum is being used instead of mitomycin C with similar efficacy and reduced toxicity.(305)

4.3.8 ANAL SCC STAGING

Anal cancer is staged by the tumour, node, and metastasis classification. (288) (See Table 4.1)

4.3.9 ANAL SCC PROGNOSIS

The prognosis of anal cancer with treatment is dependent upon the depth of invasion and the extent of spread of the tumour at diagnosis.(306) (290) (307) (308) (309) (310) Five year survival rates vary from 91% (T1), 80% (T2), 16% (T3) and 0% (T4).(307) Additionally, recurrence rates when initial tumour has infiltrated muscle or pelvic soft tissue is 60%.(306) There is no prognostic significance in the following pathological and molecular factors: keratinization, HPV presence or absence, mitotic rate, invasive margin, and cell pleomorphism.(311) (310) (312) HIV infection does not appear to have a negative impact on prognosis.(313)

Table 4.1: Tumour, node, metastasis staging of anal cancer

Abbreviation	Definition
Primary tumour	
TX	Primary tumour cannot be assessed
TO	No evidence of primary tumour
Tis	Carcinoma in situ (=AIN-3)
T1	Tumour 2 cm or less in the greatest dimension
T2	Tumour 2-5 cm in the greatest dimension
T3	Tumour exceeding 5 cm in the greatest dimension
T4	Tumour of any size invading adjacent organ
Regional Lymph Nodes	
NX	Regional Lymph nodes cannot be assessed
NO	No regional lymph node metastases
N1	Metastases in the perirectal lymph node(s)
N2	Metastases in unilateral internal iliac and/or inguinal lymph node(s)
N3	Metastases in perirectal and inguinal lymph nodes and/or bilateral internal iliac lymph nodes and/or inguinal lymph nodes.
Distant Metastases	
MX	Presence of distant metastases cannot be assessed
MO	No distant metastases
M1	Distant metastases

T: tumour, N: node, M: metastasis

4.4 STUDY AIM

The aim of this retrospective study was to investigate the demographics of individuals diagnosed with anal cancer in the San Francisco Bay Area that had prior cytological or pathological evidence of high-grade anal dysplasia.

4.5 METHODS

This study was performed with the approval of the University of California San Francisco (UCSF) Committee on Human Research (CHR). A chart review was undertaken of all cases of anal SCC diagnosed or treated at UCSF that had prior anal dysplasia documented by either cytology or biopsy. Subjects diagnosed with anal cancer who had no prior pathological evidence of anal dysplasia were excluded from the analysis. Referral sources included the UCSF Anal Neoplasia Study,(206) the UCSF Dysplasia Clinic, and clinicians both at UCSF and elsewhere within the San Francisco Bay Area. Charts were accessed at UCSF central records and also at referring locations. All cytology and tissue specimens in this study were retrieved and reviewed in the UCSF Department of Pathology (TD).

4.6 RESULTS

Eight subjects were found who met the criteria for inclusion in this case series.

Subject 1: Anal biopsies were performed consequent to a perianal abscess in January 1996 and showed internal AIN 3. In May 1996 anal fissures were biopsied and showed AIN 3 at both perianal and internal anal sites. Anal cytology was not obtained. In December 1996 an anal canal mass was biopsied and confirmed as

invasive anal cancer with AIN 3 reported at other internal anal and perianal biopsy sites. (See Table 4.2)

Table 4.2: Subject 1

	1/96	5/96	12/96
ANAL CYTOLOGY			
EXTERNAL ANAL BIOPSY		AIN 3	SCC + AIN 3
INTERNAL ANAL BIOPSY	AIN 3	AIN 3	AIN 3

LSIL: low grade squamous intraepithelial lesion, HSIL: high-grade squamous intraepithelial lesion, AIN-1: anal intraepithelial neoplasia grade 1 (equal to low-grade dysplasia), AIN 2 and 3: anal squamous intraepithelial neoplasia grade 2 and 3 (equal to high-grade dysplasia), SCC: squamous cell cancer.

Subject 2: Anal biopsy in March 1996 showed internal AIN 2 and 3 and external circumferential AIN 3. Anal cytology was not obtained. He was followed without treatment. Further internal anal biopsies in January 1997 showed internal AIN 2/3 at three locations. In March 1999 he developed a gastric B cell lymphoma that was treated by gastrectomy in May 1999, delaying further evaluation of his anal disease. In June 1999 anal cytology showed HSIL, an internal anal biopsy showed high-grade dysplasia and an external biopsy showed superficially invasive SCC. (See Table 4.3)

Table 4.3: Subject 2

	3/96	1/97	6/99
ANAL CYTOLOGY			HSIL
EXTERNAL ANAL BIOPSY	AIN 3		SCC
INTERNAL ANAL BIOPSY	AIN 2 and 3	AIN 2/3	AIN 2/3

Subject 3: Perianal AIN 3 was diagnosed by biopsy but untreated in March 1996. Anal cytology showed HSIL in March 1997. In August 1997 an examination under anesthesia with biopsies confirmed internal anal SCC and AIN 2/3. (See Table 4.4)

Table 4.4: Subject 3

	3/96	3/97	8/97
ANAL CYTOLOGY		HSIL	
EXTERNAL ANAL BIOPSY	AIN 3		
INTERNAL ANAL BIOPSY			SCC + AIN 2/3

Subject 4: Anal cytology showed HSIL in May 1996. The subject reported a new internal anal nodule in August 1996, when cytology showed HSIL and nodule biopsy showed AIN 2/3. He was referred for a surgical opinion where biopsy of an ulcerating internal anal mass in November 1996 confirmed moderately differentiated SCC. (See Table 4.5)

Table 4.5: Subject 4

	5/96	8/96	11/96
ANAL CYTOLOGY	HSIL	HSIL	
EXTERNAL ANAL BIOPSY			
INTERNAL ANAL BIOPSY		AIN 2/3	SCC

Subject 5: Anal cytology in August 1999 showed HSIL with internal biopsies showing AIN 1 (indicating the biopsy missed the high-grade lesion). He was seen in December 1999 with a superficial anal ulcer when cytology again showed HSIL and an internal anal biopsy confirmed SCC. (See Table 4.6)

Table 4.6: Subject 5

	8/99	12/99
ANAL CYTOLOGY	HSIL	HSIL
EXTERNAL BIOPSY		
INTERNAL BIOPSY	AIN 1	SCC

Subject 6: Anal cytology taken in December 1997 showed LSIL. Subsequent cytology showed: ASCUS in May 1998; LSIL in August 1998; HSIL in December 1998; and HSIL in June 1999 when the first internal anal biopsy was taken and showed AIN 2. He presented in April 2000 with a two-month history of bleeding per rectum, tenderness and a protruding mass. SCC was diagnosed by cytology and internal anal biopsy. (See Table 4.7)

Table 4.7: Subject 6

	12/ 97	5/ 98	8/98	12/ 98	6/ 99	4/00
ANAL CYTOLOGY	LSIL	ASCUS	LSIL	HSIL	HSIL	SCC
EXTERNAL BIOPSY						
INTERNAL BIOPSY					AIN 2	SCC

Subject 7: Circumferential Bowen's disease was diagnosed in March 1994 and treated with 5-fluorouracil cream. He subsequently had circumferential Bowen's disease diagnosed on biopsy in 1995 and 1997 and was treated with tri-weekly Imiquimod from February 1999 to August 1999. In March 1999 anal cytology showed LSIL. In May 2000 an internal anal biopsy showed AIN 2, and in February 2000 cytology showed HSIL. He was seen with anal pain in May 2000 and surgery was recommended. Surgical biopsies taken in June 2000 confirmed invasive well-differentiated SCC at 3 sites in the anal canal. (See Table 4.8)

Table 4.8: Subject 7

	3/94	95	97	3/ 99	5/ 00	2/ 00	6/ 00
ANAL CYTOLOGY				LSIL		HSIL	
EXTERNAL BIOPSY	AIN 3	AIN 3	AIN 3				
INTERNAL BIOPSY					AIN 2		SCC

Subject 8: Anal cytology taken in February 1997 showed HSIL, confirmed by biopsies in April 1997, which showed extensive internal AIN 2/3 and external anal AIN 2/3 with a Bowenoid appearance. At that time she underwent a simple vulvectomy for vulval intraepithelial neoplasia (VIN), followed by laser treatment to the vulva/perineum and perianal area in July 1997. Further anal cytology showed ASCUS in December 1997 and squamous metaplasia in July 1998. AIN 2/3 was reported on internal anal biopsy in August 1998 and perianal superficially invasive SCC and AIN 2/3 with introital VIN 1 in March 1999. (See Table 4.9)

Table 4.9: Subject 8

	2/ 97	4/97	12/97	7/ 98	8/98	3/ 99
ANAL CYTOLOGY	HSIL		ASCUS	SQ MET		
EXTERNAL BIOPSY		AIN 2/3				SCC
INTERNAL BIOPSY		AIN 2/3			AIN 2/3	

Sq met.: Squamous metaplasia. (Laser treatment to perineum/perianal was performed at the time of a simple vulvectomy for VIN in July 1997)

Seven of the eight subjects (83%) were male. The mean age of the subjects at the time of anal cancer diagnosis was 45.1 years with a range of 39 to 54 years. Accurate CD4 counts and HIV viral loads at time of diagnosis were not available. The mean time from first diagnosis of high-grade dysplasia by anal cytology or biopsy to the diagnosis of anal cancer was 14.4 months, with a range of 3 to 23 months. Five of eight subjects (63%) had a history of anal warts, three (37.5%) had history of recreational drug use, two (25%) had not used recreational drugs, and recreational drug history was unknown for three (37.5%) subjects. Three (37.5%) subjects were current smokers, two (25%) were former smokers, one (12.5%) subject had no history of smoking and the smoking history was unknown for two (25%) subjects. All male

subjects were MSM, had been HIV-positive for over 10 years and were taking highly active antiretroviral therapy (HAART) at the time of diagnosis. The one female subject was heterosexual and HIV-negative. (See Table 4.10)

Table 4.10: Subject demographics

SUBJECT	HIV	CD4 NADIR	REC. DRUGS	SMOKER	STI HX	HX. ANAL WARTS	AGE AT DIAG.	HSIL TO CANCER
1	POS	0	NO	PREVIOUS	YES	NO	44y	11 months
2	POS	119	YES	PREVIOUS	YES	YES	54y	37 months
3	POS	176	YES	CURRENT	YES	YES	39y	5 months
4	POS	143	UNK	CURRENT	UNK	YES	44y	6 months
5	POS	241	YES	NO	NO	NO	40y	3 months
6	POS	UNK	UNK	UNK	UNK	YES	54Y	17 months
7	POS	130	UNK	UNK	UNK	YES	42y	13 months
8	NEG	NA	NO	CURRENT	NO	NO	44y	23 months

Pos: positive, Neg: negative, NA: not applicable, UNK: unknown, Hx: history, Diag: diagnosis, Rec: recreational, STI: sexually transmitted infection

4.7 DISCUSSION

4.7.1 PROGRESSION OF HIGH-GRADE ANAL DYSPLASIA TO ANAL SCC

These cases demonstrate that anal SCC develops in the presence of high-grade squamous intraepithelial lesions diagnosed by either anal cytology or anal biopsy, and is consistent with the hypothesis that high-grade anal dysplasia may be a precursor lesion to the development of anal cancer. Three cases also confirm the previously reported progression of external high-grade anal dysplasia to anal SCC.(314)

AIN 2/3 likely takes several years to progress to invasive anal cancer, as is known to be true of the temporal relationship between high-grade cervical dysplasia and the development of cervical cancer.(315) The data in this study show that the mean time

from first diagnosis of high-grade dysplasia by anal cytology or biopsy to the diagnosis of cancer was 14.6 months. However this figure likely underestimates the true transition time to cancer as incident high-grade disease was only captured in 2 cases. In these cases the incident high-grade dysplasia diagnosis was made on anal cytology which although sensitive for detection of anal dysplasia is poorly predictive of anal dysplasia grade, with most cytology samples indicating lower grades of dysplasia than are confirmed by anal biopsy. Thus, it is feasible that high-grade dysplasia was present prior to its' cytological diagnosis. All other subjects had high-grade dysplasia at first cytological or pathological diagnosis. In the two incident cases the interval from first high-grade dysplasia diagnosis to cancer was 29 months and 13 months. Clinical experience in the Anal Neoplasia Study suggests that this is an unusually short timeframe for progression, as many subjects have been observed with high-grade anal dysplasia without treatment for many years. Thus it is unlikely that the data permit an accurate estimation of time to progression to anal cancer from first developing high-grade dysplasia. They do show, however that in this high-risk population of predominantly HIV-positive MSM, anal cancer can occur within a relatively short period of time after first clinical detection of high-grade dysplasia.

4.7.2 DEMOGRAPHICS OF STUDY SUBJECTS

The demographics of subjects with anal cancer in the general population differ from those described in this study by having a female predominance and a peak incidence of anal cancer diagnosed in the seventh decade.(288) (316) At diagnosis, study subjects were predominantly male, with an average age of 45 years. All male subjects were HIV-positive with an average known seropositivity of 13 years. It is noteworthy

that previous reports of anal cancer incidence including the SEER database do not include HIV serostatus in the demographic analysis.(279)

4.7.3 EFFECT OF HIV INFECTION AND HAART

Although HIV infection in MSM may have been expected to lead to an increase in the risk of anal cancer, an increase beyond that observed in HIV-negative MSM was not detected during the early years of the HIV epidemic.(275) This may have been due to individuals succumbing to opportunistic infections or other HIV-related malignancies before anal cancer had time to develop. Consistent with this postulate, subjects in this study developed anal cancer at a mean of 13 years after the first documented positive HIV test while median time interval to an AIDS diagnosis from HIV seroconversion in an untreated population is on average 10 years.(317)

All of the HIV-positive subjects reported having taken HAART at some time prior to, or at the time of, the diagnosis of anal cancer. Little is known about the effect of HAART on the incidence of anal cancer, although recent studies in San Diego and San Francisco showed an increased incidence in the post HAART era.(278) (279) In Europe, Bower *et al* concluded that since the introduction of HAART there had been no significant change in the incidence of anal cancer at a central London teaching hospital cohort of HIV infected individuals.(277)

If progression from anal HSIL to anal cancer takes many years, individuals taking HAART with its positive impact on survival may now have sufficient time for progression to occur. Thus, in the absence of routine screening and treatment of anal HSIL, individuals taking HAART may paradoxically be at increased risk of developing anal cancer.

4.7.4 PROGRESSION OF INDIVIDUAL HIGH-GRADE DYSPLASTIC LESIONS

It is clear from studies in San Francisco and elsewhere that there is a higher prevalence of high-grade anal dysplasia diagnosed than anal cancer, similar to high-grade cervical dysplasia and cervical cancer.(315) Assuming high-grade dysplasia is the precursor lesion to anal SCC, there is no current method of detecting exactly which area of high-grade dysplasia will progress to cancer. Thus, the detection of high-grade anal disease should lead to consideration of ablative treatments or close clinical monitoring.

4.7.5 STUDY LIMITATIONS

This study has several noteworthy limitations. The study was performed as a retrospective chart review and so was limited to available charts and documentation with the potential that cases were missed that were not present in the UCSF pathology database. Additionally data available were often sparse and did not include a comprehensive medical history and investigation with chronological details of antiretroviral therapy, sexual history, smoking start and stopping dates, in addition to those parameters shown in Table 4.10.

The awareness of anal dysplasia screening and availability of both studies and services was likely to be more apparent to the MSM community in the San Francisco Bay area and so may have biased the sex ratio. However, initial data from the UCSF pathology database prior to exclusion of subjects without prior documentation of anal high-grade dysplasia also showed a male predominance.

It cannot be certain that the anal cancers arose in the exact site where the high-grade dysplasia was diagnosed when anal biopsy data was present, as dysplasia may have

been multi-focal. Additionally some biopsy locations were simply documented empirically as internal or external. However, even if there is no direct relationship between previously identified high-grade dysplasia and the subsequent development of anal cancer, detection of high-grade dysplasia may at least represent a marker for an individual at risk of developing cancer.

4.8 SUMMARY

This study indicates that anal cancer may be diagnosed subsequent to previous documentation of high-grade anal dysplasia. HIV-positive MSM subjects developed anal cancer at a younger age than the general population.

Large prospective cohort studies are required to determine the efficacy of anal cytology screening to identify anal dysplasia, and treatment of high-grade dysplasia to prevent anal cancer. However, these studies will be difficult to perform because of the large numbers of patients required, the length of time required for follow-up, and the complex ethical issues involved. It is noteworthy that implementation of cervical cancer screening programs preceded unequivocal evidence that a cytolopathological diagnosis of dysplasia and its' ablation led to a reduction in cervical cancer incidence.

CHAPTER 5

THE SENSITIVITY AND SPECIFICITY OF SELF-TAKEN ANAL CYTOLOGY SAMPLES FOR THE DIAGNOSIS OF ANAL SQUAMOUS INTRAEPITHELIAL LESIONS IN MEN WHO HAVE SEX WITH MEN

5.1 INTRODUCTION

The incidence of anal cancer in the United States is rising. To address this, investigators have used anal canal cytology screening as a means of detecting anal dysplasia, with subsequent ablation of high-grade dysplasia similar to the screening and treatment paradigm utilized for the female cervix as has previously been discussed in Chapter 2 and 3. Several barriers currently exist to the implementation of an anal cytology screening program for high-risk individuals. One obstacle is the lack of studies to document that treatment of HSIL reduces the incidence of anal cancer. Performance of such studies is hampered by the large number of subjects needed, difficulties in assembling an appropriate control group and the long follow-up time needed to assess the impact on cancer incidence as discussed in Chapter 4. Other obstacles include a paucity of clinicians trained in collecting anal cytology and trained in HRA, the potential reluctance of clinicians to address the issue of ASIL with their patients, and patients' feelings of embarrassment and fear of potential discomfort associated with the collection of anal specimens. Collection of anal cytology samples by patients themselves has the potential to obviate some of these concerns. It has previously been shown that women can successfully obtain samples of cervicovaginal fluid at home for HPV testing.(318) This method was not adapted to cervical cytology screening because of the anatomic challenges in blindly directing the sample collection devices precisely where they are needed in the cervix. However, the anal canal is an easier anatomic site for self-directed collection of cells.

The purpose of this study was to assess the performance of results from anal cytology samples collected by study participants at home compared to samples collected by experienced research clinicians in a clinic of MSM with a high prevalence of ASIL.

Anal histopathology was used as the gold standard for this research comparison. The published paper is presented in Appendix 1.

5.2 METHODS AND MATERIALS

5.2.1 SUBJECTS

The study was performed with the approval of the UCSF Committee on Human Research. Informed consent was obtained from all subjects. Participants were recruited from 505 HIV-positive and 364 HIV-negative MSM in the UCSF Anal Neoplasia Study.(196)

5.2.2 CLINIC ANAL CYTOLOGY AND BIOPSY SAMPLING

Study participants were examined every 3-6 months with anal cytology and HRA with biopsy of any lesion suspicious for dysplasia. Demographic information was collected and blood samples were obtained for HIV antibody testing.

One hundred twenty five study participants from the ongoing Anal Neoplasia Study population were sequentially invited to participate in this study. Criteria for study eligibility included having had an anal cytology sample taken using glass-slide smears and ethanol fixation, and a simultaneous biopsy of any visible anal lesion that appeared dysplastic using HRA. The study participants had previously had anal cytology samples collected by their practitioner. The men were consecutively chosen for participation during the study period, and neither the cytology nor the biopsy results were known at the time of enrollment. A clinician provided an explanation of the study, and informed consent was obtained from study subjects. Cytology

specimen collection and HRA were performed as described previously with standard glass slide and ethanol fixation technique used for clinician-collected samples.(196)

5.2.3 HOME ANAL CYTOLOGY SAMPLING

The study participants used the liquid cytology method to collect their specimens at home, as they have similar performance characteristics to glass-slide cytology (189) but are substantially easier to handle. The men were given written instructions on how to take an anal cytology sample. The collection kit consisted of: one bottle of Cytyc ThinPrep (Cytyc Corp., Boxborough, MA) sample collection medium, one Dacron swab, two sealable plastic bags for disposal and a pair of latex gloves. To allow for healing of the initial anal biopsy site, subjects were requested to wait one month from the time of the original anal cytology and biopsy prior to collecting their cytology sample.

While wearing latex gloves, the men were instructed to remove the Dacron swab from its sterile package and moisten it with tap water before inserting it two inches into the anal canal. Applying gentle lateral pressure to the walls of the anal canal, participants were instructed to remove the swab with a spiral motion over ten seconds. The swab was to be immediately placed into an open Cytyc bottle containing Cytyc ThinPrep media and agitated vigorously to disgorge the cells from the swab before it was removed from the bottle. Participants were instructed to close the Cytyc bottle tightly, store it at room temperature and return it to the clinic in a sealed plastic bag within one week of specimen collection.

5.2.4 CYTOLOGY SAMPLE ANALYSIS

The same pathologist analysed all clinician collected cytology and biopsy samples. Because of the different sampling method used, the pathologist knew whether the specimen was clinician-collected or self-collected. However, all specimen findings were interpreted without reference to the paired specimen. Cytology findings were classified according to the Bethesda classification system as inadequate for interpretation (sample contained less than 2000 to 3000 nucleated squamous cells), negative for squamous intraepithelial lesion, ASCUS, LSIL, or HSIL. At the time of this study, the new Bethesda 2001 terminology of ASCH had not been developed and so does not appear in any diagnostic category.

5.2.5 STATISTICAL ANALYSIS

Statistical analysis was performed using Version 8 of the Statistical Analysis System (SAS Inc., Cary, NC). The sensitivity of anal cytology to detect LSIL and HSIL was defined as the number of specimens with abnormal cytology divided by the number of individuals with AIN. The Fishers exact test was used to compute p values for comparing sensitivities or other proportions across groups.

The Fishers exact test is an eponymous statistical significance test used in the analysis of categorical data. The test is used to examine the significance of the association between two categorical variables in an r x c contingency table, where one variable has "r" categories and the other has "c" categories. A special case is a 2 x 2 table (r=c=2). With large samples or well-balanced tables, a chi-square test p value is a good approximation for the exact Fisher p value. However, the chi square approximation is no longer accurate when the sample size is small or the expected

frequencies in any cell of the table is below 5 or the expected frequency is below 10 in a 2 x 2 table.

In the context of the above study, the Fishers test was chosen, as this is the exact method for computing a p value when comparing two independent proportions/percentages for a binary outcome. For example, the Fisher procedure is the correct way to compute a p value for comparing the 3/15 = 20% response in 15 HIV negative patients versus the 56/73 = 77% response in 73 HIV positive patients as these are clearly two independent groups. A patient must be HIV negative or HIV positive and cannot be both. If one is comparing percentages/proportions where two different medical tests or determinations are compared on the same (paired) samples or patients, then the Fisher test is not the appropriate way to compute a p value.

The observed p value is a function of the data. Using $p < 0.05$ (where 0.05 is the alpha level) is a generally accepted criterion for deciding whether the p value is 'statistically significant'.

5.3 RESULTS

One hundred and six MSM subjects who had a clinician-collected anal cytology and HRA examination with anal biopsy were enrolled from the UCSF Anal Neoplasia Study. Nineteen subjects either declined to take part in the study or did not return the cytology samples in a timely manner. Four subjects without complete self-collected cytology, clinic collected cytology and biopsy results were excluded from the analysis. Of the 102 remaining subjects, 82 were HIV-positive and 20 were HIV-negative. The mean age at time of sampling was 45 years (range 29-72 years). Most of the subjects were white non-Hispanic, n = 89 (87%); the rest were white-Hispanic,

n = 2 (2%); African American, n = 3 (3%); Native American n = 1 (1%); Asian, n = 3 (3%); and other, n = 4 (4%).

5.3.1 RESULTS OF CLINICIAN-COLLECTED AND SELF-COLLECTED ANAL CYTOLOGY, AND CORRESPONDING ANAL HISTOLOGY

The results of clinician-collected anal cytology and the corresponding histopathology are shown in Table 5.1. The results of the self-collected anal cytology and the corresponding histopathology are shown in Table 5.2. Specimen adequacy was high in both the clinician-collected (101/102, 99%) and the self-collected cytology (93/102, 91%) with a significantly higher rate of adequacy seen in the clinician-collected samples (p=0.02). The adequacy rate of self-collected samples was similar for HIV-positive (93%) and HIV-negative (85%) men (p=0.37).

Table 5.1: Comparison of clinician-collected anal cytology with clinic biopsy

	Anal histopathology					
Clinician-collected cytology	**Normal**	**Atypical**	**AIN 1**	**AIN 2 or 3**	**Insufficient**	**Total (%)**
Normal	4	1	10	18	0	**33 (32%)**
ASCUS	1	0	0	2	0	**3 (3%)**
LSIL	0	1	13	22	0	**36 (35%)**
HSIL	0	0	2	27	0	**29 (28%)**
Insufficient	0	0	1	0	0	**1 (1%)**
Total (%)	**5 (5%)**	**2 (2%)**	**26 (26%)**	**69 (68%)**	**0 (0%)**	**102**

Insufficient: insufficient cellular material for interpretation, AIN 1: low-grade anal intraepithelial neoplasia, AIN 2 or 3: high-grade anal intraepithelial neoplasia)

Table 5.2: Comparison of self-collected anal cytology with clinic biopsy

	Anal histopathololology					
Self-collected cytology	**Normal**	**Atypical**	**AIN 1**	**AIN 2 or 3**	**Insufficient**	**Total (%)**
Normal	3	1	8	20	0	**32 (31%)**
ASCUS	0	0	1	3	0	**4 (4%)**
LSIL	0	1	10	22	0	**33 (32%)**
HSIL	0	0	1	23	0	**24 (24%)**
Insufficient	2	0	6	1	0	**9 (9%)**
Total (%)	**5 (5%)**	**2 (2%)**	**26 (26%)**	**69 (68%)**	**0 (0%)**	**102**

5.3.2 SENSITIVITY OF CLINICIAN-COLLECTED AND SELF-COLLECTED ANAL CYTOLOGY TO DETECT ANY BIOPSY PROVEN ABNORMALITY

The sensitivity of any grade of anal cytology abnormality for detection of AIN 1, AIN 2, or AIN 3 was comparable between the clinician-collected (70%) and self-collected (68%) samples. The sensitivity of any grade of anal cytology abnormality for detection of a high-grade lesion (i.e., AIN 2 or 3) was also compared between the clinician-collected (74%) and self-collected (71%) samples.

5.3.3 THE EFFECT OF HIV SEROSTATUS ON COLLECTION OF ANAL CYTOLOGY SAMPLES

The sensitivity of anal cytology to detect AIN in self-collected samples was higher among HIV-positive men than among HIV-negative men. Three (20%) of the 15 HIV-negative men with AIN had abnormal cytology compared with 56 (77%) of 73 HIV-positive men ($p<0.01$). The sensitivity for detection of AIN 2 or 3 specifically in self-collected specimens was also higher among HIV-positive men than among HIV-negative men. Among the 57 HIV-positive men with biopsy proven AIN 2 or 3, anal

cytology was abnormal in 43 (75%), compared with 4 (40%) of 10 HIV-negative men (p=0.05). The sensitivity of anal cytology to detect either AIN overall or AIN 2 or 3 specifically was also higher for HIV-positive men in the clinician-collected samples, and the magnitude of these differences in sensitivity was similar to those seen in self-collected samples (p=0.15 for AIN overall; p=1.0 for AIN 2 or 3). Overall cytologic results did not differ by grade between clinician-collected and self-collected samples. Among men diagnosed with AIN 2 or 3 by biopsy, 39% with AIN 2 and 33% with AIN 3 had HSIL by cytology.

5.4 DISCUSSION

In this study, we compared overall rates of abnormality between samples collected by clinicians and study participants. The results of this study strongly suggest that in this population of MSM, with previous experience of having had anal swabs used to collect cytology specimens and anal biopsies of lesions clinically suspicious for anal dysplasia taken by a clinician, are capable of self-collecting samples with sensitivity comparable to experienced clinicians. This was accomplished with only written instructions on how to self-collect an anal cytology specimen in this experienced population.

5.4.1 ANAL CYTOLOGY SCREENING

Recent guidelines from the United States Public Health Service for the treatment of opportunistic infections in HIV-positive individuals indicate that although anal cytology screening has not yet been formally recommended, anal cytology screening should be considered for HIV-positive men and women. Anal cytology should be used as a screening tool to identify individuals who would benefit from HRA to detect

and treat HSIL.(196) The current UCSF recommendation is to refer patients with any grade of anal cytologic abnormality for HRA.(114) Moreover the UCSF group previously showed that detection of HSIL on anal cytology had a high positive predictive value for detection of biopsy-proven high-grade dysplasia.(196) Thus, inability to confirm high-grade dysplasia on HRA-directed biopsy in a patient with HSIL cytology should prompt a repeat examination.

5.4.2 ANAL CYTOLOGY SENSITIVITY

The primary goal of anal cancer prevention is to detect and treat HSIL before it progresses to cancer. To this end, we also compared the sensitivity of cytology collected by clinicians and subjects to detect AIN 2 or 3 by biopsy and found that the sensitivity of the two groups was comparable. This ability to self-collect anal cytology may allow high-risk populations to be screened outside of a medical setting, and allow for subsequent evaluation with HRA and treatment of lesions in individuals with abnormal cytology. It could also provide a useful tool for epidemiologic studies of anal cytology and possibly anal HPV in large population-based cohorts.

5.4.3 ANAL CYTOLOGY SPECIMEN ADEQUACY AND HIV

The rates of clinician-collected and self-collected anal cytology specimen adequacy were similar to those seen in a previous study that validated anal cytology as a screening test for ASIL.(196) In the present study, the adequacy of specimens collected by clinicians was slightly higher than those collected by study subjects. However, this should not affect the role of self-screening since patients with inadequate specimens could be informed of the need to repeat the test. Notably the sensitivity of anal cytology to detect anal dysplasia overall and AIN 2 or 3 specifically

was significantly higher among HIV-positive men than HIV-negative men, a finding consistent with an earlier study.(196) We speculate that this reflects the larger size of lesions typically found in HIV-positive men, leading to a higher likelihood of detection. Another explanation is that HIV-negative men were not as vigorous in obtaining specimens as the HIV-positive men. This is less likely because adequacy rates for specimens were similar between the HIV-positive and HIV-negative men. Further, the clinician-collected specimens were less sensitive among the HIV-negative men than the HIV-positive men, similar to our findings for the self-collected specimens. Because participants were enrolled in the study only if they had undergone anal biopsy, the prevalence of ASIL in the study was very high, and there were too few participants with normal anoscopic examination findings to assess differences in other measures of screening validity (eg, specificity and positive and negative predictive values) between sample specimens. In a predominantly HIV-negative population we would expect fewer abnormal anal cytology results, consistent with the less frequent diagnosis of anal dysplasia in this population. However, despite the statistically insignificant differences in specimen adequacy between the HIV-positive and HIV-negative subjects, there were fewer HIV-negative participants in this study and further investigation of this population would be appropriate.

5.4.4 FALSE NEGATIVE ANAL CYTOLOGY

As with previous studies of anal and cervical cytology, a substantial proportion of normal cytology specimens were false negative. In this study approximately 30% of subjects with biopsy-proven ASIL had normal cytology suggesting that, similar to cervical screening, sequential anal cytology screening may be necessary to detect ASIL in this population. In this context, the role of testing for high-risk HPV as an

adjunct to anal cytology has not been characterised. Previous studies have shown that detection of multiple HPV types is a risk factor for progression of LSIL to HSIL, although the risk of progression associated with individual high-risk HPV types has not been fully addressed.(206)

5.4.5 STATISTICAL CONSIDERATIONS

Fishers exact test is used for computing p values when comparing proportions (sensitivity is an example of a proportion) in different categories. All of the above data is categorical and is expressed as two way categorical cross tabulations. Before the advent of easy to use computational software, approximate p values for comparing proportions in different categories were computed with the chi-square test method. However, computer software such as SAS has been available for the last 10 years or so that performs the exact p value computation using Fishers method, making the chi-square approximation no longer necessary.

The statistical power/sample size in the above study may be too low, however, this does not invalidate the Fisher p value computation method but is a separate criticism of inadequate sample size, a criticism that could be made regardless of the statistical computation procedure. Additionally, examining many associations by doing many p value calculations for many statistical tests is a problem in any exploratory study. Again, this is not a shortcoming of the Fisher test; it is a shortcoming in any exploratory study where many potential associations are examined. The problem with examining many potential associations or retesting after changing the definitions is that one of the tests may come out "significant" as an artifact of the multiple testing, that is a "false positive". In the above study we compared physician collected vs self collected and HIV positive vs HIV negative and we considered both AIN overall and

AIN 1 vs AIN 2-3 separately. Since we examined less than a dozen associations or redefinitions, multiple significance testing is a not a major problem in this study. Further, observational studies frequently tend to be exploratory in nature and results usually are reported without an alpha correction.

Additionally, the above study did not investigate the efficacy of anal cytology sampling by clinicians or subjects with no previously diagnosed anal pathology, and was therefore unable to report specificity of anal cytology sample in the two groups studied.

5.4.6 STUDY LIMITATIONS

The results of self-collected anal cytology were encouraging in this study, although the results should be interpreted with caution. The study participants in this study had experienced the collection of multiple anal cytology samples by clinicians in the past and were aware of how an adequately collected sample felt while being taken. This experience may have enhanced their ability to take an adequate specimen. Self-collected samples were taken one month following clinic anal cytology and anal biopsy. It is possible, although unlikely given the slow progression of dysplastic lesions in the cervical and anal canal that the degree of dysplasia may have changed during this time. It is possible, although also unlikely, that taking an anal biopsy could have completely removed the dysplastic lesion or changed the natural history of the lesion. Clinician-collected cytology specimens were obtained using a glass slide and were preserved in ethanol, whereas self-collected specimens were processed using liquid cytology methods. The liquid method may have facilitated improved specimen adequacy by reducing processing problems associated with faecal contamination and low cell count. However, when previously assessed using a split-sample technique,

both methods were shown to be comparable, although this may not have been the case in this study.(189)

One of the strengths of this study was that the same pathologist analysed all of the cytology and biopsy specimens, facilitating comparison of the results. The pathologist was also aware if the cytology was clinician or subject collected because of the different sampling methods used. Therefore, a potential bias may have been introduced into the interpretation of cytology results. However, we do not believe that this affected the results in a substantive way because the pathologist interpreted the samples at least 1 month apart and would not know the findings for the other samples collected from the same participant.

5.5 SUMMARY

In summary, these data indicate that self-collected anal cytology specimens, when adequate for interpretation, had similar sensitivity to specimens collected by experienced clinicians. The sensitivity of anal cytology to detect AIN 2 and 3 is good among HIV-positive men, but further effort is needed to improve sampling among HIV-negative men. If successful, this technique could greatly facilitate institution of anal cytology screening to prevent anal cancer among at-risk individuals and would be a useful tool to define the prevalence of abnormal anal cytology in large populations. Further research is needed to determine whether this technique works as well when performed by individuals with no prior experience with anal cytology and among a population with a lower prevalence of AIN. In addition, the sensitivity, specificity, and positive and negative predictive values should be studied for populations with a lower prevalence of AIN and with a larger number of HIV-negative men.

CHAPTER 6

ANOGENITAL CYTOKINE BIOLOGY IN HEALTH AND DISEASE

6.1 INTRODUCTION

This chapter will discuss the biological properties of selected cytokines and their involvement in infectious and neoplastic disease with particular reference to HPV associated disease in the cervical canal, in the absence of current data for the anal canal.

6.2 CYTOKINES

Cytokines are transcriptionally regulated proteins secreted by T helper (Th) cells and other cell types in response to a variety of stimuli, and function by acting on specific cell receptors to regulate the host immune response. They may act in an autocrine or paracrine manner, binding to cell receptors on the cell of origin or those in the immediate vicinity respectively. Cytokines may additionally act on cells distant to the cells of origin analogous to the action of a hormone.

6.2.1 Th1/Th2 PARADIGM

The T helper type 1 (Th1) and Th type 2 (Th2) nomenclature of cytokines was first used by Mosmann and Coffman in 1986 when reporting that murine Th lymphocyte clones could be divided into two subsets based on the function of the immunoregulatory cytokines secreted by these cells; Th1 cells (interferon gamma (IF-γ), interleukin 2 (IL-2), granulocyte macrophage colony stimulating factor (GM-CSF) and interleukin 3 (IL-3)) and Th2 cells (interleukin 4 (IL-4), interleukin 5 (IL-5), interleukin 6 (IL-6), interleukin 10 (IL-10) and GM-CSF)).(319) Th1 cytokines are pro-inflammatory and stimulate cell-mediated immunity (CMI) that is effective in the host defense against viral infections and tumour development. Th2 cytokines

stimulate B-lymphocyte development and antibody production against extracellular pathogens.(320) Th2 cytokines are anti-inflammatory or immunoinhibitory and may facilitate tumour growth.(321) This classification was extended to humans in 1991 (322) and following this observation the pathogenesis of infectious, inflammatory and neoplastic disease has been classified by predominant cytokine phenotype.(323) (324) (325) (320)

More recently it has been established that T cells may secrete both Th1 and Th2 cytokines and are labeled Th0 (or Th0-like).(326) (327) Early cytokine contact and the nature of the antigen signal influence differentiation of Th0 cells into either Th1 or Th2 effectors.

There is one further immunoregulatory T cell type that co-expresses CD4 and CD25 (the IL-2 receptor alpha chain) on the cell surface and are phenotypically immunosuppressive.(328) These cells have been labeled Treg although this function is not limited to this particular cell type. It is apparent that Treg cells are critical in the development of the immune response in autoimmune disease and hypothetically, if down regulated, may function to augment tumour development.(329)

6.2.2 CYTOKINE NOMENCLATURE

Th1 and Th2 cytokines are produced by a diverse lineage of cell types that include CD4 T cells, CD8 T cells, monocytes, NK cells, B cells, eosinophils, mast cells, basophils and epithelial cells.(330) (331) More recently the terms 'Th1-like' and 'Th2-like' are being used to reflect the diversity of cell types that can produce cytokines.(330) For simplicity, the following two chapters will use Th1 to represent Th1-like cytokines and Th2 to represent Th2-like cytokines.

6.2.3 CYTOKINE BIOLOGY

6.2.3.1 IF-γ

The IF-γ gene is located on chromosome 12. IF-γ is the dominant Th1 cytokine secreted by activated T lymphocytes and NK cells.(332, 333) IF-γ increases cell surface expression of major histocompatability complex (MHC) class I antigens, increases cell surface expression of MHC class II antigens on antigen presenting cells, augments tumour necrosis factor (TNF) cytotoxicity, and activates macrophages resulting in enhanced killing of intracellular pathogens.(334) (335) (336) There is minimal homology between IF-γ and the two other interferons (IF-α and IF-β) as IF-γ binds to a separate cell receptor.(337)

6.2.3.2 IL-2

The IL-2 gene is located on chromosome 4.(338) IL-2 is a Th1 cytokine secreted by activated T lymphocytes and was first identified as a T cell growth factor in 1976.(339) (332) IL-2 stimulates proliferation and differentiation of T lymphocytes by autocrine and paracrine up-regulation of the high-affinity α/β IL-2 receptor. It also enhances B cell growth and the cytolytic effect of NK cells in addition to increasing IL-4 and IF-γ production by other lymphocytes.(340) (341)

6.2.3.3 IL-4

IL-4 is a Th2 cytokine secreted by T lymphocytes, macrophages, mast cells, basophils, B lymphocytes, bone marrow and epithelial stromal cells.(332) Its' action promotes the differentiation of CD4 T lymphocytes into Th2 cells and suppresses Th1

differentiation. It also induces the proliferation and differentiation of B lymphocytes, and the production of immunoglobulin (Ig) E.(341, 342)

6.2.3.4 IL-10

IL-10 is a Th2 cytokine secreted by T lymphocytes, macrophages, keratinocytes, B lymphocytes, and epithelial cells.(332) (343) It acts to suppress the functional activity of macrophages, and inhibits both macrophage and monocyte production of pro-inflammatory cytokines. It also increases B lymphocyte proliferation, MHC class II expression and immunoglobulin (Ig) secretion.(344) IL-10 inhibits IL-12 production by dendritic cells and monocytes.(345) IL-10 m-RNA and protein has been found in excess in several cancers, and been shown to be produced by tumour infiltrating lymphocytes and tumour cells.(346-349)

6.2.4 CYTOKINES AND THE MAJOR HISTOCOMPATABILITY COMPLEX

The MHC determines the body's response to antigen in that the peptide/MHC complex presented to the T-cell will help determine the Th1 or Th2 response to antigenic stimulation. This has been demonstrated in both human and murine studies and may be particularly important in the context of chronic infections where the response to the pathogen is vital to host defense and prognosis.(350) A Th2 response is associated with down-regulation of MHC class I and β_2-microglobulin expression and so reduces antigen presentation to antigen presenting cells, whose function is concomitantly impaired in this environment.(351) (352) (353) (354)

6.2.5 CYTOKINE GENE EXPRESSION IN THE FEMALE GENITAL TRACT

Cytokine gene expression has been detected by RT-PCR in the female genital tract from cervico-vaginal secretions that, in health, exhibit a predominantly Th1 type environment.(355) This is not the case in the cervix of HIV-positive women in the absence of detectable HPV or CSIL who show a predominantly Th2 type cytokine gene expression pattern.(356)

Female genital tract cytokine gene expression has been investigated in the context of HPV infection. Female subjects with cervical intraepithelial neoplasia (CIN)/anogenital HPV and a predominantly Th1 type cytokine response from *in vitro* stimulated PBMC's have more contained HPV disease than subjects with a predominantly Th2 type cytokine response.(321) This finding was also demonstrated by Stellato *et al* who reported that high serum IL-2 levels may predict a better prognosis in women treated for genital HPV associated lesions.(357) The mechanism of this finding may in part relate to the *in vitro* finding that IL-10 increases the transcription of HPV E7 in HPV 16-positive cervical cancer cell lines.(358)

Local cervical cytokine gene expression has also been investigated by RT-PCR from material collected by cervical swabs in subjects infected with HPV. This study by Scott *et al* demonstrated that all subjects who cleared HPV infection, as evidenced by a negative HPV PCR, had a preceding Th1 cytokine gene expression pattern and subjects with persistent HPV lacked a Th1 response.(359)

A comparison was reported between Th1 and Th2 cytokine production in women with cervical LSIL and HSIL, using matched controls. PBMC and whole blood culture cytokine levels of IL-4 and IF-γ showed no significant difference between the two groups, however, an inverted relationship of IL-12 and IL-10 with a Th2 predominance was found in subjects with cervical HSIL.(360)

The cytokine milieu of cervical tissue has been less frequently reported in the literature. Al-Saleh *et al* analysed transition zone and ectocervical biopsy specimens from normal and dysplastic cervices. Immunohistochemistry was used to detect cells secreting IL-2, IF-γ, IL-4, and IL-6. Higher densities of Th1 cytokines were found in normal tissue and higher densities of Th2 cytokines were found in CSILs. The authors suggested that this immunomodulation may participate in cervical carcinogenesis.(361) Similar findings were reported by El-Sherif *et al* using quantitative RT-PCR from paraffin embedded cervical specimens.(333)

6.2.6 CYTOKINE GENE EXPRESSION IN CERVICAL CANCER

Cytokine gene expression is considered part of the host defense to injury or disease, and as such may reflect disease activity.(362) Serum IL-2 and production of IF-γ by activated PBMC's is reduced in subjects with invasive cervical cancer, and additionally may be reduced at the tumour site in poor prognosis cases. (363-365) IF-γ has been shown to suppress expression of HPV E6 and E7 genes in both immortalized and cervical cancer cell lines, as well as enhancing peptide presentation to cytotoxic T-cells.(366) (367) Although a reduction of Th1 and enhancement of Th2 activity had been suggested as a response to, or mediator of, the progression of cervical dysplasia to cancer, not all investigators have demonstrated this, with one study reporting a generalised impairment of cervical cancer T cells and down-regulation of both cytokine classes.(368)

6.2.7 CYTOKINE GENE EXPRESSION AND CONCURRENT SEXUALLY TRANSMITTED INFECTION

The anogenital tract is prone to both sexually transmitted and non-sexually transmitted infection (STI). The cytokine environment of the cervical canal, with particular reference to IL-10 gene expression, has been studied in women with STI's with conflicting findings. Hedges *et al* report that infection with *T. vaginalis*, *C. trachomatis* and *N. gonorrhoea* have no influence on IL-10 gene expression (369), whereas Cohen *et al* reported that IL-10 was isolated more frequently in women with concurrent STI.(370)

6.2.8 CYTOKINE GENE EXPRESSION IN THE ANAL CANAL

The cytokine environment of the anal canal has not previously been investigated. Sampling techniques that have been used to investigate the cervix include biopsy and exfoliative cytology. Collection of anal cytology specimens is less invasive when compared to anal biopsy, although the collected material is hypocellular and predominantly composed of anal squamous cells although immune cells such as T lymphocytes may also be present (S. Hirschowitz – personal communication).

6.2.9 CYTOKINE GENE EXPRESSION AND HIV INFECTION

Infection with HIV-1 results in a progressive destruction of the cell-mediated immune system with profound loss of CD4 lymphocytes resulting in increased risk of opportunistic infection and the development of certain malignancies. The immune milieu during chronic HIV infection and progression to AIDS has been studied with particular reference to Th1 and Th2 cytokine gene expression, and various theories presented to better understand the clinical decline of these patients. Clerici and

Shearer have proposed a dysregulation of cytokine gene expression with a shift from Th1 to Th2 predominance to predict clinical decline.(324, 371) There was pre-existing evidence suggesting an impairment in T-cell proliferation and IL-2 production in vitro using PBMC's from immunocompetent HIV-positive patients with normal CD4 counts.(372) The Th2 predominance does not appear to be restricted to HIV subtype B, the type most commonly seen in Western infections, with a Th2 predominance also demonstrated in other parts of the world with predominant infection with HIV subtype C and is also seen in HIV-2 infection.(373)

The Th type of immune response may be modulated by the ratio of circulating antibody/antigen complexes. Berger *et al* described a Th1 to Th2 type shift when *ex-vivo* peripheral blood mononuclear cells (PBMC) were challenged with antibody-excess immune complexes, as occurs frequently in HIV disease in the form of polyclonal hypergammaglobulinemia.(374)

In the era of highly active antiretroviral therapy (HAART), response to therapy as measured by decreasing HIV RNA in plasma and concomitant increase in CD4 lymphocyte count has been shown to increase levels of IL-2 and IF-γ while also showing a decrease in IL-10 by RT-PCR. This was reported in an antiretroviral exposed cohort commencing a new antiretroviral regime that included a class of drug to which the patient had not been previously exposed, so increasing the probability of treatment efficacy and immunological response.(375)

Long-term non-progressors are HIV patients who do not show a reduction in CD4 cell numbers over time and usually have very low or undetectable steady state untreated plasma HIV viral load. This population showed a balance in Th1/Th2 profiles without a Th2 predominance.(376) This paradigm does not appear to be limited to adults with HIV infection, with vertically HIV-infected long term non-progressing children also

displaying increased Th1 compared to Th2 cytokines, while as with adults, clinically progressing children have a Th2 predominance.(377)

6.3 SUMMARY

Cytokines are transcriptionally regulated proteins secreted by a variety of cells that orchestrate the body's immune response to infection and may influence tumour development. The cytokine phenotypes are broadly classified into a pro-inflammatory Th1 type that facilitates antigen presentation and T cell proliferation, and an immunoinhibitory Th 2 type that decreases macrophage function and promotes B cell differentiation.

In the normal cervicovaginal environment there is a predominantly Th1 type milieu that may change following infection although bacterial sexually transmitted infections do not appear to promote a consistent response. Infection with anogenital HPV is more likely to be contained when a Th1 response is detected, with the development of HPV associated dysplasia facilitated by a Th2 environment. Similarly HIV infection is predominantly associated with a Th2 type response, clinical decline and disease progression. This may be reversed to a predominantly Th 1 response when HAART is instituted. Thus the local or generalised cytokine environment may be essential to successful containment of infection and may influence tumourogenesis.

It remains unknown, given the anatomical, histological and pathological homology, if the cervical canal cytokine response is similar in the anal canal.

CHAPTER 7

DETECTION OF ANAL CANAL CYTOKINES USING THE REVERSE TRANSCRIPTION POLYMERASE CHAIN REACTION

7.1 INTRODUCTION

HPV infection is confined to stratified squamous epithelium. The host cell mediated immune response to HPV and HPV associated lesions is critical to viral clearance, or to suppress viral replication to undetectable levels, as has been demonstrated in the female cervix.(378, 379) To date there have been no reports on cytokine gene expression in the anal canal of either male or female subjects with HPV-associated lesions.

7.2 HYPOTHESIS

High-grade anal dysplasia is the putative precursor of anal cancer. Currently, there is no method available to predict which individual is more likely to progress to a higher level of dysplasia or to develop anal cancer. Assuming similarity to the female cervix, it was hypothesised that detection of high-grade anal cytological abnormality would be associated with a predominantly Th2 type local cytokine environment in the anal canal.(321)

7.3 AIMS

1. To confirm that cytokine gene expression is detectable by RT-PCR from material collected from anal swabs.

2. To compare cytokine gene expression in MSM subjects with high-grade and low-grade anal dysplasia with and without HIV infection.

7.4 METHODS

7.4.1 SUBJECTS

Subjects were recruited sequentially from the Anal Neoplasia Study at UCSF. The UCSF Committee on Human Research approved participation in the research study, and each study subject received written and verbal explanations of study procedures before signing informed consent.

The Anal Neoplasia Study is funded by the National Institute of Health and has been established at UCSF, San Francisco since 1992. The study comprises 658 MSM (407 HIV-positive and 251 HIV-negative), who are seen at 3-6 monthly intervals depending on their grade of anal dysplasia.

Prior to each clinic visit, subjects were advised to refrain from placing anything in the anal canal for at least 24 hours. An anal examination was performed which comprised taking an anal cytology swab, an anal swab for human HPV detection (typing by PCR), and high-resolution anoscopy with or without anal biopsy.

7.4.2 EXPERIMENTAL PROCEDURES

Anal swabs were collected as described above. RNA was purified from the anal swab material, and reverse transcribed to complementary DNA (cDNA). This was followed by optimised PCR amplification using primers for IF-γ, IL-2, IL-4, and IL-10. The housekeeping gene Glyceraldehydes-3-phosphate dehydrogenase (GAP-DH) was used to verify sample and cDNA integrity. To verify the authenticity of the cytokine PCR gel electrophoretic product, a Southern transfer was performed, and cytokine amplified product detected by enhanced chemiluminescence.

7.4.2.1 SPECIMEN COLLECTION

This study employed a Dacron™ (Baxter Healthcare Corporation, McGraw Park, IL. USA) swab to obtain an anal canal sample. With the patient in the left lateral position, the swab was inserted 2 inches beyond the anal margin. With firm pressure, and a spiral motion to sample the entire anal circumference, the swab was withdrawn over 10 seconds and placed immediately in 1000 µL of denaturing solution. This swab was taken following the anal cytology and HPV swabs. Any swab with evidence of frank blood on visual inspection was discarded.

7.4.2.2 DENATURING SOLUTION

A base solution was made by dissolving 250 g guanidinium thiocyanate (Roche Diagnostics Corporation. Indianapolis, IN. USA) in 293 mL molecular grade water (UCSF) and adding 17.6 mL 0.75M sodium citrate pH 7 (Fisher Scientific. Fairlawn, NJ. USA) and 26.4 mL 10% sarcosyl (Sigma Molecular Biology. St. Louis, MO. USA).(380) The solution was dissolved in a waterbath set at 60 °C for one hour then stored at room temperature. The base solution was discarded if unused after 3 months. To create the final denaturing solution, 0.36 mL of 2mercaptoethanol (Sigma molecular biology. St. Louis. MO. USA) was added to 50 mL of the base solution. The denaturing solution was divided into 1000 µL aliquots and stored at 4 °C until required. The aliquots were discarded if not used within one month.(381)

7.4.2.3 STORAGE AND TRANSPORT OF SAMPLES

Anal swabs were collected from subjects at the General Clinical Research Clinic (GCRC) at UCSF Mount Zion hospital, San Francisco and placed immediately in denaturing solution and stored at -80 °C for no more than two days. The swabs were

collected from Mt Zion hospital and transported in a sealed polystyrene container to UCSF Parnassus, San Francisco, a journey of fifteen minutes. On arrival at UCSF Parnassus the swabs were immediately placed in a -80 °C freezer for no more than seven days before processing.

7.4.2.4 PRECAUTIONS TAKEN TO AVOID RIBONUCLEIC ACID DEGRADATION

A laminar airflow sterile cabinet was cleaned with 75% ethanol (Quantum Chemical Co. Tuscola, IL. USA) and RNase-Erase™ (Krackeler Scientific Inc. Albany, NY. USA). All Gilson pipettes and Eppendorf tube racks were then placed in the cabinet and exposed to UV light for at least 15 minutes before use. Latex gloves were worn and washed in 75% ethanol prior to working in the sterile cabinet. Sterile microcentrifuge tubes and micropipette tips were used at all times, as were dedicated Gilson pipettes.

7.4.2.5 RIBONUCLEIC ACID EXTRACTION

The specimens were taken out of the -80 °C freezer, placed in the laminar air-flow sterile cabinet and allowed to thaw at room temperature for 15 minutes. Ribonucleic acid (RNA) extraction was subsequently performed by the phenol:chloroform method.(381)

Working in the sterile cabinet, the swab and denaturing solution were vortexed for 10 seconds then 900 µL of swab lysate was transferred to a labeled 2 mL Eppendorf microcentrifuge tube. One tube was filled with un-inoculated denaturing solution to serve as a negative control. 90 µL of 2M Sodium acetate pH4, and 900 µL of phenol:chloroform:isoamyl alcohol (IAA) (25:24:1) (Roche Diagnostics Corporation.

Indianapolis, IN. USA) were added to each tube. The tubes were mixed by inversion 5 times, vortexed for 2 minutes, then chilled on ice for 15 minutes. The tubes were then centrifuged at 8000 rpm for 20 minutes at 4 °C. Avoiding the interface, the upper aqueous phase was transferred to a new Eppendorf microcentrifuge tube. 800 µL of stock isopropanol 99% (Sigma Molecular Biology. St. Louis, MO. USA) was added to each sample, vortexed for 10 seconds and incubated at minus 20 °C for at least 2 hrs. The tubes were then centrifuged at 8000 rpm for 20 minutes at 4 °C and the supernatant poured off. 1 mL of 70% ethanol was added and the tubes were vortexed for 2 minutes to dissolve the pellet. The tubes were centrifuged at 8000 rpm for 20 minutes at 4 °C and the supernatant was poured off. The pellet was air dried in the sterile cabinet before re-suspending in 25 µL diethylpyrocarbonate (DEPC) (Sigma Molecular Biology. St. Louis, MO. USA) treated water and vortexed until dissolved. The RNA was stored at -80 °C until quantified by spectrophotometry and subsequently reverse transcribed.

7.4.2.5.1 OPTIMISING RNA EXTRACTION

To investigate whether the number of phenol:chloroform:IAA extractions had any impact on either the amount, concentration or purity of RNA, this step was repeated with groups of 6 randomly chosen specimens for 1 to 5 rounds of extractions.

7.4.2.6 SPECTROPHOTOMETRY

RNA was quantified by spectrophotometic determination at wavelengths of 260 nm and 280 nm. Optical density (OD) at 260 allowed calculation of RNA concentration. An OD 260 of 1 corresponds to 0.04 µg/µL of RNA. The ratio of OD 260/280

provides an estimate of purity of the nucleic acid. Ideally, the nucleic acid OD 260/280 ratios should fall between 1.8 and 2.0.

To measure the OD 260/280 ratio, 5 µL of RNA solution (from a total of 25 µL) was diluted in 495 µL DEPC treated water in a quartz cuvette. The cuvette was placed in the spectrophotometer and the measurement was taken. The qúartz cuvette was washed in DEPC treated water and shaken dry between samples. The spectrophotometer used was an Amersham GeneQuant Pro (Amersham Biosciences. Piscataway, NJ. USA).

Total RNA was calculated using the equation:

Total RNA (µg/µL) = OD 260 x dilution factor x 0.04

7.4.2.7 REVERSE TRANSCRIPTION

Single stranded cDNA is manufactured by reverse transcription of messenger RNA (mRNA) that can then be used as a template for PCR amplification. In order to limit transcription to mRNA, primers are used (random hexamers) that hybridize to the 3' polyadenylated tail of mRNA and allow the enzyme reverse transcriptase to initiate cDNA synthesis. Reverse transcriptase is derived from cloned Moloney murine leukaemia virus (MMLV). RT-PCR allows investigators to work with much smaller quantities of RNA than was previously possible using experimental methods such as the Northern blot procedure.

7.4.2.7.1 REVERSE TRANSCRIPTION REACTION

All reagents were stored at -20 °C in a designated PCR room with designated instruments and no contact with post PCR product.

The quantities for 1 reaction were: 0.2 µL of random hexamers (Promega. Madison, WI. USA) were diluted 1:5 in molecular grade water (UCSF) to give a final volume of 1 µL and kept on ice. The RT reaction mix was prepared with 4 µL 5X MMLV RT buffer (Promega. Madison, WI. USA), 3 µL 10 mM 4 dNTP mix (final concentration 1.5 mM) (Promega. Madison, WI. USA), 1.75 µL molecular grade water, 0.25 µL RNAsin Ribonuclease inhibitor 40 u/µL (Promega. Madison, WI. USA), and 1 µL MMLV 40 u/µL (Promega. Madison, WI. USA) to make a final volume of 10 µL and kept on ice.

For the primer annealing step 2 µg of total RNA were combined with 1 µL diluted random primer and a variable volume of molecular grade water (depending on RNA volume to give 2 µg of total RNA) to a final volume of 10 µL. The mix was heated to 95 °C for one minute in a Biometra1 thermal cycler, cooled to 4 °C and kept on ice.

For the RT reaction, 10 µL of RT reaction mix was added to the hexamer mix and incubated at 37 °C in the Biometra thermal cycler for 1 hour before heat inactivating the reverse transcriptase at 95 °C for 5 minutes and cooling to 4 °C. The 20 µL volume product was stored at -20 °C until used.(382)

7.4.2.7.2 REVERSE TRANCRIPTION AND PCR NEGATIVE CONTROL CONTROL

In every round of reverse transcription, one negative control was included which contained no RNA template. This sample was reverse transcribed, PCR performed and PCR product was run with all other samples on the final agarose gel.

7.4.2.8 AGAROSE GEL MANUFACTURE

1.5 percent agarose gels were made by adding 0.75 g of electrophoresis grade agarose (GibcoBRL. Gaithersburg, MD. USA) to 50 mL 10X Tris-Acetate-EDTA (TAE). 10X TAE was made with TRIS (Fisher Scientific. Fairlawn, NJ. USA) (48.46 g/L (0.4M), ethylenediaminetetraacetic acid (EDTA)-Na_2-salt (Invitrogen Life Technology. Carlsbad, CA. USA) 3.72 g/L (0.01 M), and acetic acid (Fisher Scientific. Fairlawn, NJ. USA) 12.01 g/L (0.2 M). The mixture was heated to boiling point in a microwave oven then 2.5 µL of ethidium bromide (Sigma molecular biology. St. Louis, MO. USA) 10 mg/mL was added to give a final concentration of 0.5 µg/mL. Two 12-toothed combs were inserted and the agarose was allowed to solidify at room temperature. The gel was used when it had cooled to room temperature.

7.4.2.9 AGAROSE GEL ELECTROPHORESIS

Each gel was submerged in an electrophoresis tray filled with 10X TAE. Each well was loaded with 10 µL of PCR product and 2 µL of 6X loading buffer (BioVentures Inc. Murfreesboro, TN. USA) per sample. A negative PCR control as defined above and positive cytokine control (see below) were also loaded on each gel in addition to 4 µL of 100 base-pair ladder (BioVentures Inc. Murfreesboro, TN. USA) with 2 µL of 6X loading buffer. The gel was run at 80 Volts for 30 minutes and then viewed and photographed with Polaroid film on an ultraviolet viewing box.

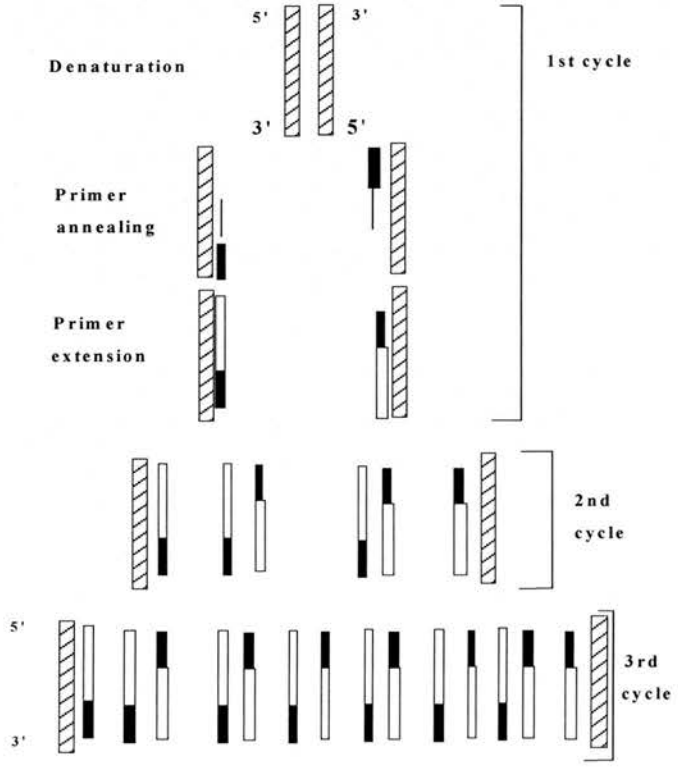
7.4.2.10 POLYMERASE CHAIN REACTION AMPLIFICATION

The basic features of the polymerase chain reaction (PCR) reaction are illustrated in Figure 7.1. PCR is a means of amplifying specific DNA sequences by the use of

complementary primer sequences and a DNA polymerase. Firstly the double stranded DNA is denatured at high temperature then cooled to allow annealing of primer sequences, present in excess, to their complementary sequences on the dissociated double stranded DNA template. After a further temperature change the DNA polymerase facilitates extension of the primer sequence in the 5' to 3' direction. The primer sequences are designed so that the extension phase will allow synthesis of regions complementary to the original primer sequences. Thus, the newly synthesized DNA can then act as a template for further cycles of amplification. The discovery of *Thermus aquaticus* (Taq) polymerase,(383) which has a half-life of 20 minutes at 94 ^{o}C, facilitated the development of automated PCR sequencing technology.

Under optimal conditions, PCR technology allows for the amplification of DNA from a single cell with an error rate calculated as 1:9000 for single base pair substitutions.(384) (385)

Figure 7.1: The polymerase chain reaction



7.4.2.10.1 PRIMER DESIGN

All primer sequences were designed so that they that spanned at least one intron/exon boundary.(See Table 7.1) An intron is a nucleotide sequence intervening between exons (coding regions) that are excised from a gene transcript during RNA processing. Thus, PCR products amplified from genomic DNA rather than cDNA could be recognised as they would be larger than the predicted size of the cDNA product. PCR product size was between 200 and 600 base pairs that allowed for clear resolution of PCR product on agarose gels without the prolonged optimization that is required for larger PCR products. Primer sequences for GAP-DH, IF-γ and IL-4 were manufactured by Operon Technologies (Alameda, CA. USA) and diluted to stock solutions of 5 pM per μL in tris[hydroxymethyl] aminomethane (TRIS) pH 8.4.

Table 7.1: Positive control and cytokine DNA primer sequences

Sequence name	Primer sequences	PCR product size (base pairs)
GAP-DH (386)	5'-C CAA AAG GGT CAT CAT CTC T-3' 5'CCT GCT TCA CCA CCT TCT TG-3'	446
IF-γ (386)	5'-AAT GCA GGT CAT TCA GAT GTA GCG G-3' 5'-GGA TGA GTT CAT GTA TTG CTT TGC G-3'	298
IL-2*	5'-CAT TGC ACT AAG TCT TGC ACT TGT CA-3' 5'-CGT TGA TAT TGC TGA TTA AGT CCC TG-3'	305
IL-4 (387)	5'-CAA CTT TGT CCA CGG ACA C-3' 5'-TCC AAC GTA CTC TGG TTG G-3'	344
IL-10*	5'-AAG CTG AGA ACC AAG ACC CAG ACA TCA AGG CG-3' 5'- AGC TAT CCC AGA GCC CCA GAT CCG ATT TTG G-3'	328

*Primer sequences supplied by Dr. Mark Scott

7.4.2.10.2 PCR REACTION CONSTITUENTS

All reagents were stored at -20 °C in a designated PCR room with designated instruments and no contact with post-PCR product.

The quantities given are for 1 reaction: The PCR reaction mixture consisted of 5 µL 10X PCR buffer (Mg free) (Promega. Madison, WI. USA), 5 µL $MgCl_2$ (various concentrations according to primer) (Perkin Elmer. Madison, WI. USA), 1 µL 10 mM 4 dNTP's (dATP, dGTP, dTTP, dCTP) (Roche Diagnostics Corporation. Indianapolis, IN. USA), 5 µL each of the 5 µM sense and anti-sense primers (Operon technologies. Alameda, CA. USA) and 24 µL molecular grade water. The total volume was 45 µL and the PCR mixture was kept on ice.(382)

The Taq reaction mixture was made with 0.2 µL Taq 5u/µL (Promega. Madison, WI. USA), 0.25 µL 10X PCR buffer (Mg free) (Promega. Madison, WI. USA) and 2.05 µL molecular grade water to a total volume of 2.5 µL and kept on ice.

The PCR reaction consisted of 45 µL of reaction mix, 2.5 µL of sample cDNA and 2.5 µL diluted Taq polymerase, to a total of 50 µL.(382)

7.4.2.10.3 PCR CYCLING PROFILE

PCR was performed on a Biometra 1 programmable thermal cycler (Whatman. Gottingen, Germany). Amplification was performed over 30 cycles (see below) consisting of denaturation at 94 °C for 30 seconds, primer annealing at 50 °C for 45 seconds, primer extension at 72 °C for 60 seconds, with the final primer extension at 72 °C for 360 seconds before cooling to hold at 4 °C. The cycling protocol was generously shared by Dr. Mark Scott.

7.4.2.10.4 PCR OPTIMISATION

7.4.2.10.4.1 RNA CONCENTRATION AND LOWER LIMIT OF DETECTION

Previous experiments performed by Dr. Mark Scott with cervical samples had suggested that a starting RNA concentration of 2 μg was adequate for RT. It was considered appropriate to confirm this for the anal samples and assess the limit of detection of the PCR. Anal swabs had RNA extracted in the usual manner and quantified by spectrophotometry. Reverse transcription was performed according to protocol on 2μg of RNA and serial dilutions were made of the cDNA to represent starting RNA concentrations of 2000 ng, 1000 ng, 500 ng, 100 ng, 50 ng, 10 ng, 2 ng.

7.4.2.10.4.2 MAGNESIUM CONCENTRATION

PCR was performed to optimise magnesium chloride concentrations with positive controls for GAP-DH, IF-γ, IL-2, IL-4, IL-10, and molecular grade water as a negative control. Magnesium chloride concentrations of 25, 20, 15, 10, and 5 mmol/L were used.

7.4.2.10.4.3 OPTIMUM THERMUS AQUATICUS CONCENTRATION

To ensure that the concentration of *Thermus Aquaticus* (Taq) was adequate for the PCR amplification, a PCR was performed using GAP-DH positive control and GAP-DH primers with Taq concentrations of 10 u, 1 u, 0.1 u, 0.01 u and 0.001 u.

7.4.2.10.4.4 PCR CYCLING PARAMETERS

Experiments were undertaken to investigate the optimum cycle number for the PCR reaction. These experiments were undertaken with GAP-DH positive control and GAP-DH primers, and used cycle numbers of 22, 24, 26, 28, 30, 32, and 34.

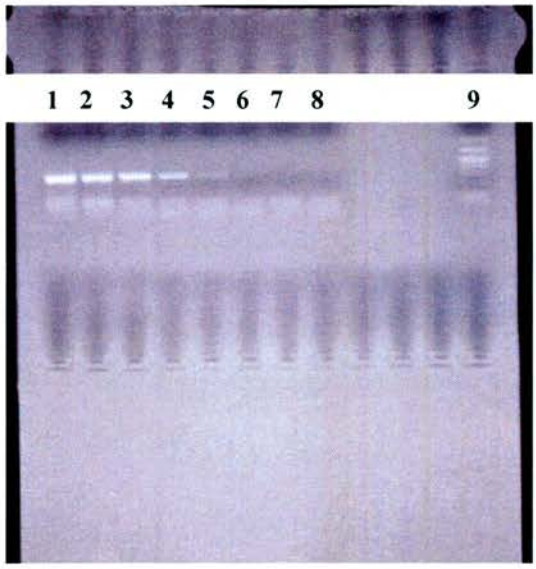
7.4.2.10.4.5 UNIFORMITY OF PCR AMPLIFICATION

In order to compare different cDNA samples, it was essential to ensure uniformity of amplication between samples. To ensure the Biometra 1 programmable thermal cycler performed in this way all 96 wells were loaded with GAP-DH positive control and amplified with GAP-DH primers. The PCR products were then run on an agarose gel.

7.4.2.10.4.6 PCR POSITIVE CONTROLS

GAP-DH is a catalytic enzyme involved in glycolysis that is constitutively expressed in almost all mammalian tissues at high levels. Reverse transcription and amplification of GAP-DH m-RNA was included in every assay as an endogenous standard. Cytokine and GAP-DH positive controls were provided by Dr Mark Scott and were derived from gel purified RT-PCR product of cervical swab material. To assess the optimal concentration of positive control for PCR, serial dilutions were made and ran on Agarose gel. (See Figure 7.2 for an example using IL-10) The clearest band with least accessory bands was chosen for each positive control, and stock solutions of this concentration were made and kept at 4 °C.

Figure 7.2: PCR of IL-10 positive control logarithmic dilutions



Lane number	PCR product IL-10 dilutions
1	1:1
2	1:10
3	1:100*
4	1:1000
5	1:10000
6	1:100000
7	1:1000000
8	1:10000000
9	100 base-pair ladder

* Dilution chosen for stock solution

7.4.2.10.4.7 VERIFICATION OF GAP-DH AND CYTOKINE PCR POSITIVE CONTROLS

Positive controls for GAP-DH, IF-γ, IL-2, IL-4, and IL-10 were amplified and the PCR product ran on an agarose gel. The gel product band for each control was excised and concentrated using a gel purification method (Quiagen Inc., Valencia, CA, USA) and subsequently sequenced by the DNA sequencing lab at UCSF.

7.4.2.11 SOUTHERN TRANSFER AND DETECTION OF PCR PRODUCT

As the PCR electrophoretic product from the anal swab samples often contained several bands in the region of the expected base-pair band, a Southern transfer was performed for cytokine band confirmation. This technique was first described by Southern in 1971 and involves transfer of electrophoretically separated fragments of DNA, after denaturation, from a gel to an absorbent sheet of material, such as nitrocellulose, to which the DNA binds. The sheet is then immersed in a solution containing a labeled probe that will hybridize to the DNA fragment.(388) Southern transfer and enhanced chemiluminescent (ECL) were performed as described below.

7.4.2.11.1 METHOD

Southern transfer was undertaken using the Trans-Blot^{R} SD Semi-Dry Electrophoretic Transfer Cell (Bio-Rad laboratories. Hercules, CA, USA).

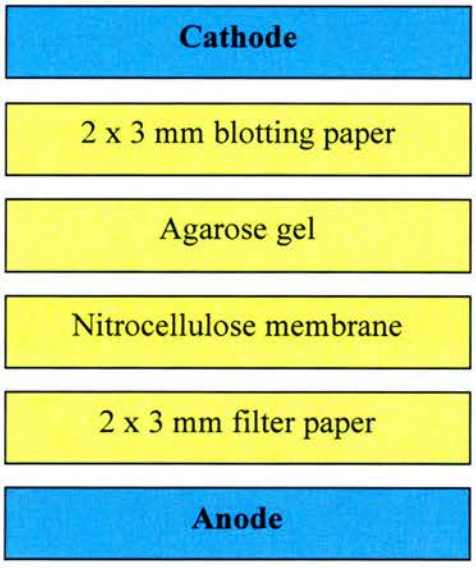
7.4.2.11.2 SOUTHERN TRANSFER

Eighteen samples of PCR product with evidence of bands corresponding to positive control for both IF-γ and IL-4 were investigated. PCR product was run with 10 μL

PCR product and 2 µL 6X running buffer on a 1.5 percent 50 mL agarose gel with 2 µL ethidium Bromide over 60 mins at 80 volts as previously described. A Polaroid phototograph was taken.

A 3 mm thick blotting paper, a nitrocellulose membrane cut slightly smaller than the agarose gel, and the agarose gel was soaked in 0.5X TAE for 15 minutes. An electrophoresis gel sandwich was assembled in an electrophoresis box as shown below. See Figure 7.3.

Figure 7.3: Southern transfer electrophoresis diagram



Any air bubbles were removed by expression, as they would impair transfer of the DNA from the gel to the nitrocellulose membrane. The cathode was set in place and the cover closed. The 50 mL gel transfer was performed at 10 volts for 30 minutes. The current calculation used was 5.5 mA/cm^2 for the 50 mL gel used.

Following transfer the membrane was rinsed briefly in 0.5X TAE buffer. DNA was fixed to the membrane by placing it on filter paper pre-soaked with 0.4 M NaOH

(Fisher Scientific. Fairlawn, NJ. US) for 10 mins. Thereafter, the membrane was rinsed in 2X standard sodium citrate (SCC) (Fisher Scientific. Fairlawn, NJ. US) for 10 mins and vacuum baked for 2 hrs at 80 °C. The membrane was then stored in a sealed plastic bag at room temperature.

7.4.2.11.3 PEROXIDASE BLOCK

The membrane was soaked in 3 % hydrogen Peroxide solution. (Fisher Scientific. Fairlawn, NJ. US) at room temperature for 15 minutes, then washed in 0.1X saline, sodium phosphate, EDTA (SSPE) (Fisher Scientific. Fairlawn, NJ. US)/0.5% Sodium dodecyl sulphate (SDS) (BioRad, Hercules, CA, USA) for 30 mins at 65°C. The membrane was then either hybridised or stored in 2X SSPE at 4°C.

7.4.2.11.4 HYBRIDISATION

The membrane was placed in a sealed plastic bag with 10 mL of the hybridization solution (5X SSPE/0.1% SDS) that had been previously warmed in a water bath to 50 °C, and 2 μL of the cytokine probe (IF-γ and IL-4 (both 5 pmol/μL) were used separately) (Operon Technologies. Alameda, CA, USA). All air bubbles were removed from the bag, it was heat sealed, and hybridisation was allowed to take place overnight at 50 °C. See Table 7.2 for details of cytokine probe sequences.

Table 7.2: Cytokine probe sequences used for hybridisation after Southern transfer

Cytokine probe	Probe sequence	TM (°C)
IF-γ	5'XTCTGTCACTCTCCTCTTTCCAATT3'	61.5
IL-4	5'XGGTGCACAGAGTCTTCTGCTCTGTG3'	67.75

X= biotin, Tm = melting temperature.

7.4.2.11.5 ENHANCED CHEMILUMINESCENT DETECTION

The membrane was removed from the hybridisation bag, immersed in a biotin wash and shaken for 30 minutes at 55 °C. A second biotin wash was performed for 30 min at 55 °C. The membrane was then immersed in a horseradish peroxidase (HRP) (Vector Laboratories, Burlingame, CA, USA) wash (5 μL of HRP added to 200mL of 37 °C wash), and shaken at room temperature for 30 min. This step was then repeated once. The membrane was removed from the HRP wash and blotted dry. The ECL was performed by adding equal quantities of each reagent and incubating the membrane for 1 minute. The membrane was then again blotted dry.

In a dark-room the dry membrane was placed next to an unexposed X-ray plate for 3 minutes and then the X-ray film was exposed using a Hope Micro-Max Model 319 developer (Hope X-Ray Products, Warminster, PA).

7.5 RESULTS

7.5.1 RNA PURITY

Spectrophotometric analysis indicated that RNA quality was less than optimal for subsequent RT-PCR. An ideal 260/280 ratio is above 1.8, and most specimen ratios averaged 1.4 (See Table 7.3). This is most likely related to the nature of the specimens, and faecal protein contamination in particular. Repeated rounds of phenol:chloroform:IAA RNA extraction did not improve specimen purity. (See Table 7.4 and Table 7.5)

Table 7.3: Sample of RNA spectrophotometry results

Sample	OD 260	OD 280	RNA concn. (µg/µL)	260/280 OD
1	0.025	0.015	0.100	1.667
2	0.032	0.019	0.128	1.684
3	0.101	0.168	0.404	1.485
4	0.413	0.281	1.652	1.470
5	0.243	0.221	0.972	1.100
6	0.220	0.141	0.880	1.560
7	0.327	0.211	1.308	1.550
8	0.225	0.151	0.900	1.490
9	0.264	0.179	1.056	1.475
10	0.434	0.298	1.736	1.456
11	0.116	0.082	0.464	1.415
12	0.192	0.130	0.768	1.477
13	0.439	0.280	1.756	1.568
14	0.277	0.182	1.108	1.522
15	0.110	0.071	0.440	1.549
16	0.354	0.236	1.416	1.500
17	0.114	0.080	0.456	1.425
18	0.129	0.086	0.516	1.500
19	0.554	0.331	2.216	1.674
20	0.975	0.668	3.900	1.460
21	0.881	0.578	3.524	1.524
22	0.712	0.489	2.840	1.456
23	0.315	0.242	1.260	1.302
24	1.277	0.867	5.108	1.473
25	1.002	0.669	4.008	1.498
26	1.432	0.914	5.728	1.567
27	2.076	1.249	8.304	1.662
28	1.193	0.806	4.772	1.480
29	1.215	0.821	4.860	1.480
30	0.715	0.504	2.860	1.419
31	1.630	1.023	6.520	0.586
32	0.914	0.636	3.656	0.437
33	0.890	0.624	3.560	1.426
34	1.642	1.048	6.568	1.567
water	0.004	0.001	0.0	4
average	0.631	0.421	2.522	1.438

OD: optic density

Table 7.4: Spectrophotometric analysis of 1-5 RNA extractions from batches of 6 anal swabs using the phenol:chloroform:IAA method

Number of extractions	OD 260	OD 280	RNA concn. (µg/µL)	260/280 OD
1	0.092	0.064	0.368	1.437
1	0.064	0.040	0.256	1.600
1	0.204	0.136	0.816	1.500
1	0.090	0.059	0.360	1.525
1	0.042	0.021	0.168	2.000
1	0.079	0.057	0.316	1.386
2	0.075	0.053	0.300	1.415
2	0.002	0.001	0.008	2.000
2	0.088	0.059	0.352	1.492
2	0.078	0.051	0.312	1.529
2	0.042	0.027	0.168	1.556
2	0.031	0.017	0.124	1.824
3	0.019	0.012	0.076	1.583
3	0.094	0.062	0.376	1.516
3	0.070	0.045	0.280	1.556
3	0.089	0.064	0.356	1.391
3	0.065	0.044	0.260	1.477
3	0.161	0.118	0.644	1.364
4	0.094	0.067	0.376	1.403
4	0.040	0.031	0.160	1.290
4	0.078	0.060	0.312	1.300
4	0.100	0.061	0.400	1.639
4	0.119	0.079	0.476	1.506
4	0.089	0.062	0.256	1.435
5	0.202	0.137	0.808	1.474
5	0.038	0.026	0.152	1.462
5	0.103	0.066	0.412	1.561
5	0.050	0.033	0.200	1.515
5	0.018	0.011	0.072	1.636
5	0.041	0.027	0.264	1.519
water	0.001	0.000	0.004	0.001

Table 7.5 Summary of mean RNA OD 260, RNA concentration, and RNA purity from samples in Table 7.4

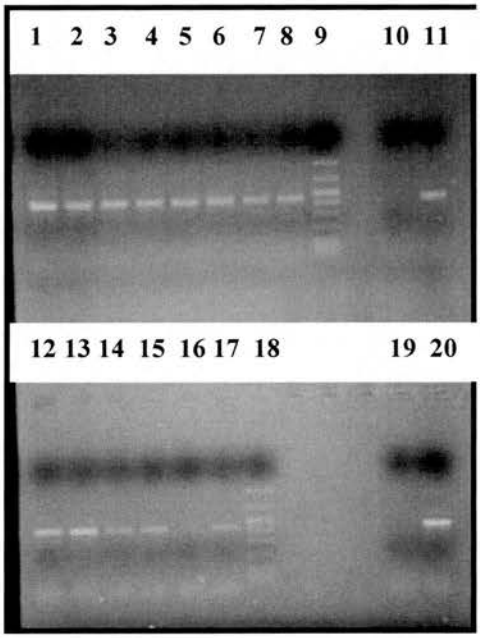
No. of extractions	Mean OD 260	Mean RNA conc. µg/µL	Mean 260/280 OD
1	0.095	0.380	1.575
2	0.053	0.212	1.636
3	0.083	0.332	1.481
4	0.087	0.348	1.429
5	0.075	0.300	1.528

7.5.2 PCR OPTIMISATION

7.5.2.1 RNA CONCENTRATION AND LOWER LIMIT OF DETECTION

The lower limit of detection for GAP-DH using GAP-DH primers for both samples was 2 ng of starting RNA. (See Figure 7.4) As GAP-DH is expressed in high concentration in human cells, it was elected to maintain the starting RNA concentration at 2 μg for the cytokine studies as it was assumed that they would be present in much lower concentrations.

Figure 7.4: GAP-DH lower limit of detection

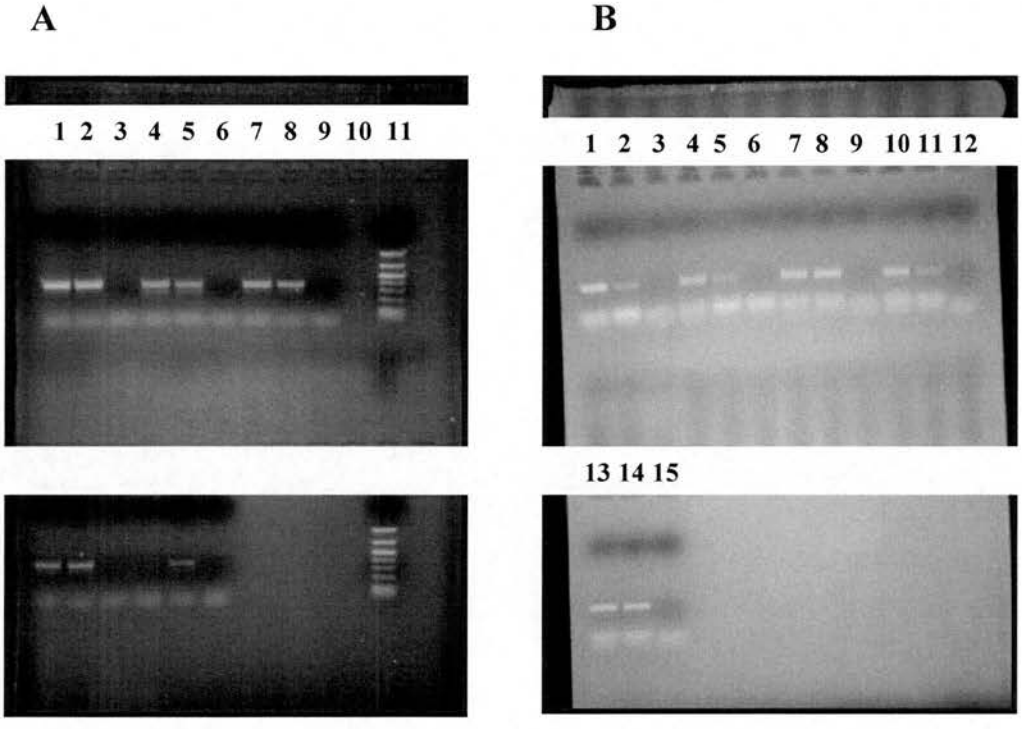


Lane Numbers	Quantification of starting RNA prior to RT and cDNA dilutions (ng)
1, 2	2000
3, 4	1000
5, 6	500
7, 8	100
12,13	50
14,15	10
16,17	2
9,18	100 base-pair ladder
11,20	Positive control
10,19	Negative control

7.5.2.2 PCR REACTION MAGNESIUM CONCENTRATION

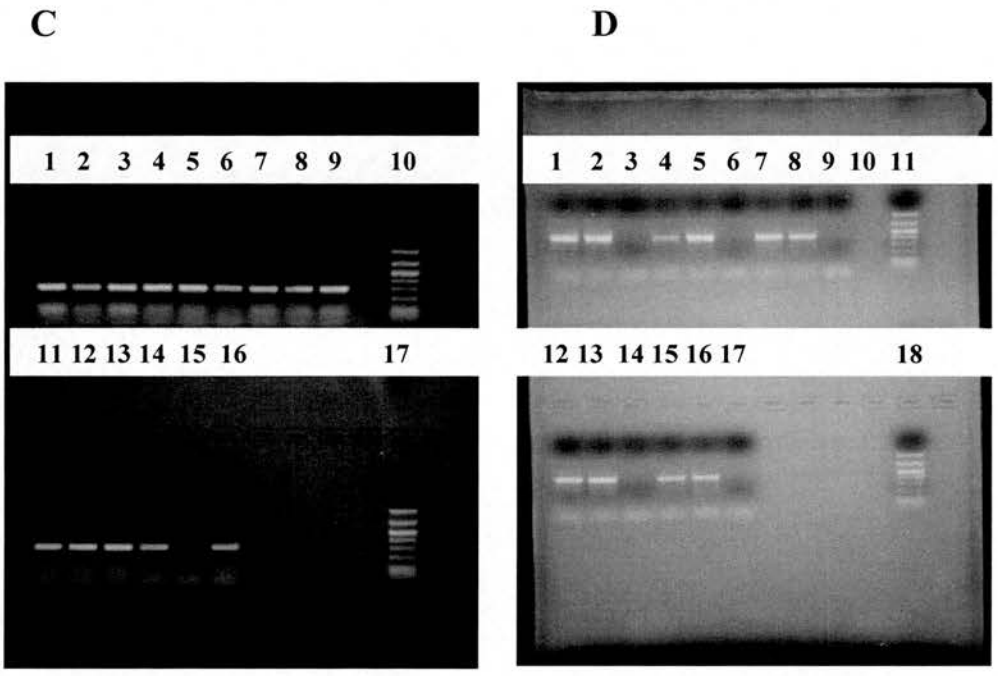
Results are shown in Figure 7.5.1, Figure 7.5.2, Figure 7.5.3, Table 7.6, and Figure 7.6 for optimum magnesium chloride concentration in PCR reactions using primers for GAP-DH, IF-γ, IL-2, IL-4, and IL-10. (* indicated the $MgCl_2$ concentrations chosen for PCR reaction mixtures).

Figure 7.5.1: Optimising PCR $MgCl_2$ concentration for A: IL-4 and B: IL-2



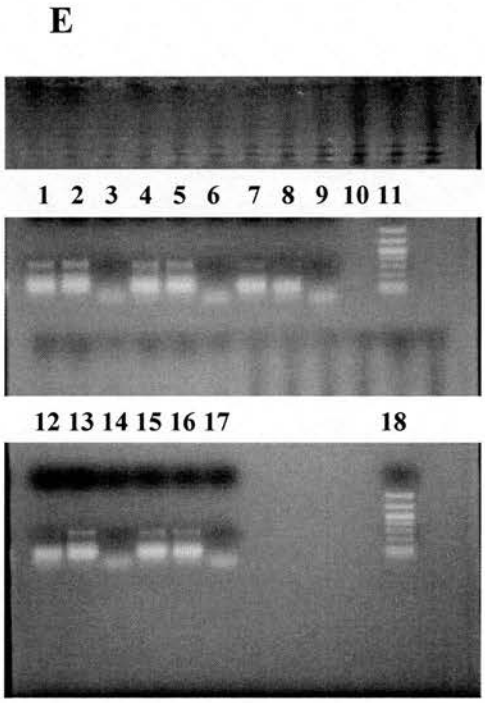
Lane Number (A)	$MgCl_2$ conc. (mmol/L)	Lane Number (B)	$MgCl_2$ conc. (mmol/L)
1, 2	25	1, 2	25
4, 5	20	4, 5	20
7, 8	15*	7, 8	15*
12, 13	10	10, 11	10
15, 16	5	13, 14	5
3, 6, 9, 14, 17	Negative controls	3, 6, 9, 12, 15	Negative controls
11, 18	100 base-pair ladder		

Figure 7.5.2: Optimising PCR $MgCl_2$ concentration for C:IF-γ, and D:GAP-DH



Lane Number (C)	$MgCl_2$ conc. (mmol/L)	Lane Number (D)	$MgCl_2$ conc. (mmol/L)
1, 2, 3	25	1, 2	25
4, 5, 6	20	4, 5	20
7, 8, 9	15*	7, 8	15
11, 12, 13	10	12, 13	10*
14, 15, 16	5	15, 16	5
10, 17	100 base-pair ladder	11, 18	100 base-pair ladder
		3, 6, 9, 14, 17	Negative controls

Figure 7.5.3: Optimising PCR $MgCl_2$ concentration for E: IL-10

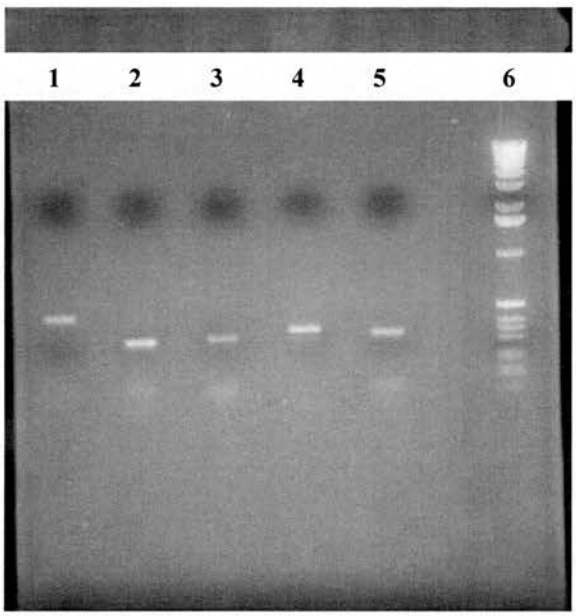


Lane Number (E)	Mg conc. (mmol/l)
1, 2	25*
4, 5	20
7, 8	15
12, 13	10
15, 16	5
11, 18	100 base-pair ladder

Table 7.6: Optimum magnesium chloride concentration for cytokine and GAP-DH PCR reactions

Positive PCR control	Optimum $MgCl_2$ concentration (mmol/L)
GAP-DH	10
IF-γ	10
IL-2	15
IL-4	15
IL-10	25

Figure 7.6 Agarose gel of optimum PCR magnesium chloride concentration for cytokine and GAP-DH positive control PCR reactions

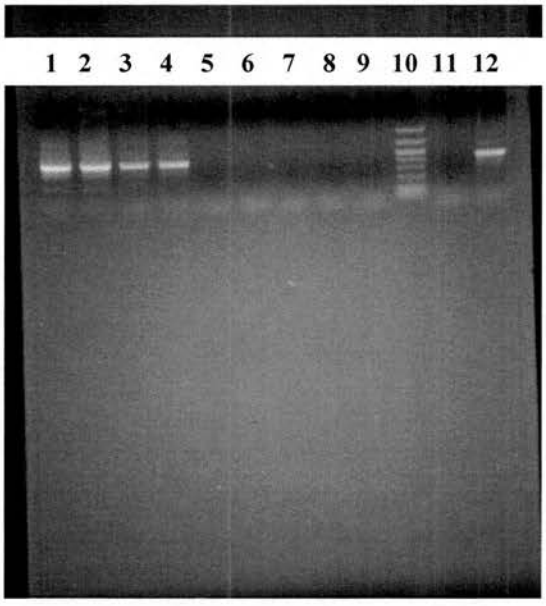


Lane number	Positive control
1	GAP-DH
2	IF-γ
3	IL-2
4	IL-4
5	IL-10
6	100 base-pair ladder

7.5.2.3 OPTIMUM THERMUS AQUATICUS CONCENTRATION

The optimum Taq concentration chosen for all PCR reactions was 1u as shown in Figure 7.7.

Figure 7.7 Optimum Taq concentration for PCR using GAP-DH positive control primer sequences



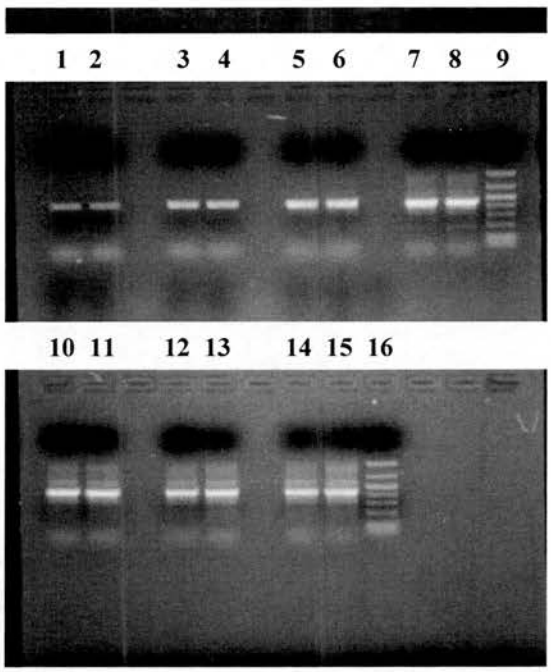
Lane Number	Taq units per PCR reaction
1	10
2	1*
3	0.1
4	0.01
5	0.001
10	100 base-pair ladder
11	Negative control
12	Positive control

* 1 unit of Taq was chosen for the PCR reactions

7.5.2.4 OPTIMUM PCR CYCLING PARAMETERS

The PCR signal for GAP-DH that was most clear with the least number of accessory bands present was seen at 30 cycles of amplification. (See Figure 7.8)

Figure 7.8 Optimum PCR cycle number



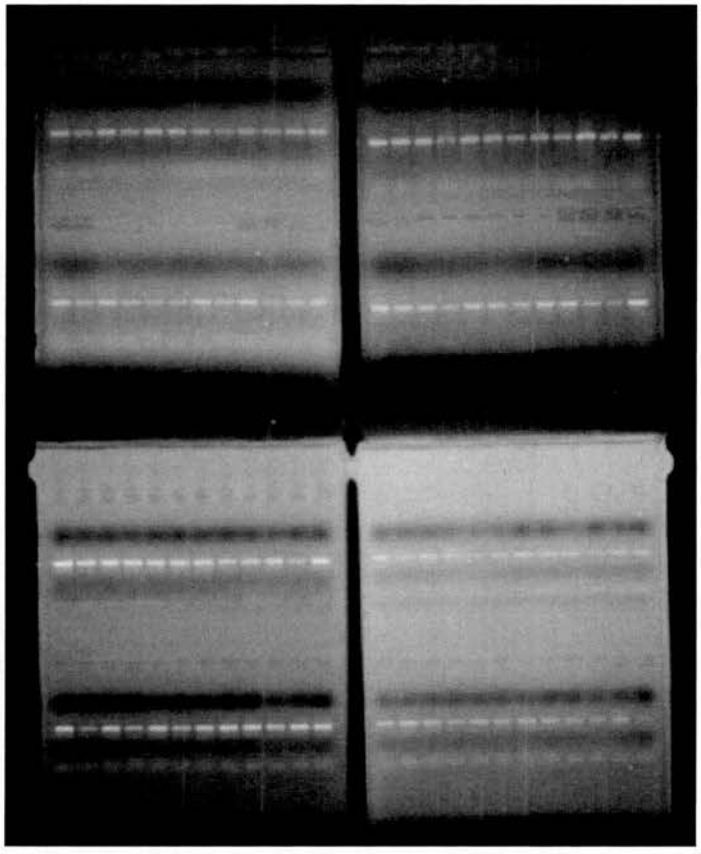
Lane Number	PCR cycle number
1, 2	22
3, 4	24
5, 6	26
7, 8	28
10, 11	30*
12, 13	32
14, 15	34
9, 16	100 base-pair ladder

* 30 cycles of PCR were chosen for the PCR reaction

7.5.2.5 UNIFORMITY OF PCR AMPLIFICATION

The Biometra 1 PCR thermal cycler showed equal amplification performance in each well using GAP-DH positive control. (See Figure 7.9)

Figure 7.9: Uniformity of PCR amplification using GAP-DH positive control



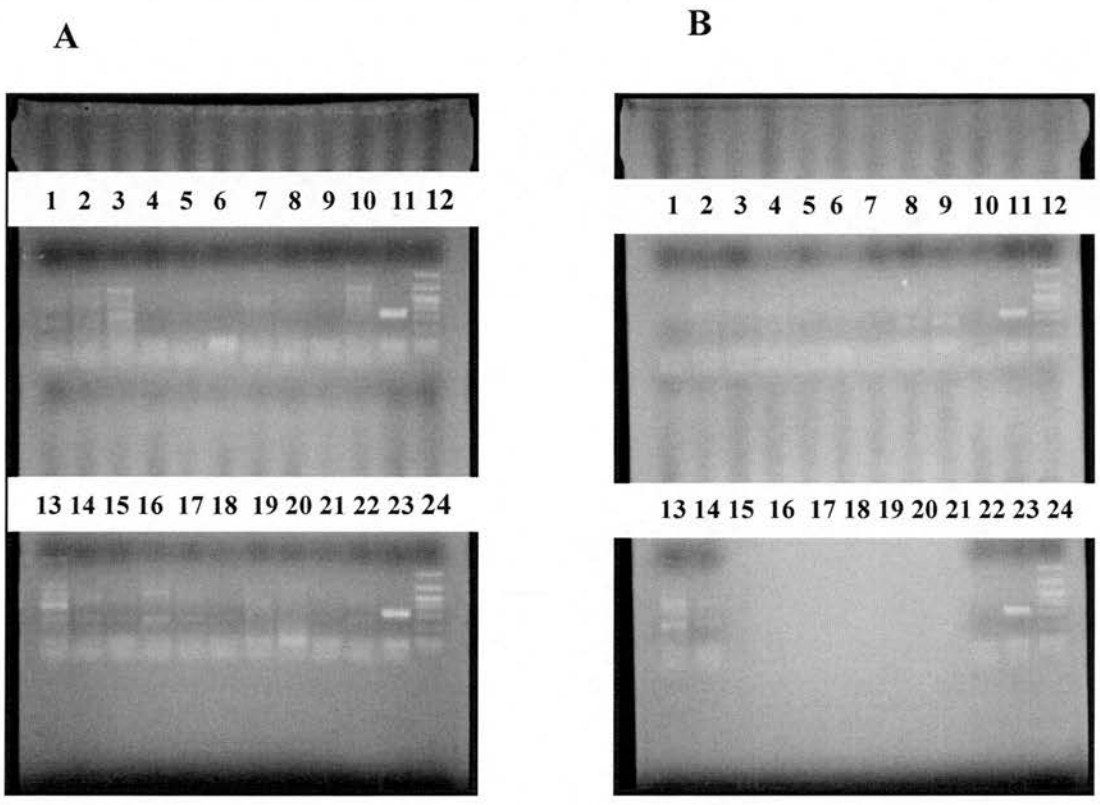
7.5.2.6 VERIFICATION OF GAP-DH AND CYTOKINE PCR POSITIVE CONTROLS

DNA sequence analysis using the basic local alignment search tool (BLAST) that provides a method for rapid searching of nucleotide and protein databases confirmed the identity of DNA sequence fragments as IF-γ, IL-2, IL-4, IL-10, and GAP-DH. (Data not shown)

7.5.3 PCR GEL ELECTROPHORESIS

Each 50 mL agarose gel was made with two 12-slot combs per anal swab batch. Each row had a positive control, and graduated 100 base-pair ladder with remaining spaces for PCR product. A single negative control was run per batch. Typically when gels were run GAP-DH was mostly positive but cytokine analysis frequently showed absent bands, or numerous accessory bands and very few visible bands that corresponded to the size of the cytokine positive control. (See Figure 7.10 as an example with positive controls for IL-10). The accessory bands were considered to reflect impurity of RNA obtained after phenol:chloroform:IAA extraction. To confirm that bands corresponding to the positive control, and to identify possible bands that may not have been visible, Southern transfer was carried out with probes for IF-γ and IL-4, representing a Th1 and Th2 cytokine.

Figure 7.9: Typical agarose gel from anal specimens using primers and positive controls for IL-10



Lane Number (A)	Contents	Lane Number (B)	Contents
1-10, 13-22	Anal swab samples	1-10, 13,14	Anal swab samples
11, 23	Positive control	11,23	Positive control
12, 24	100 base pair ladder	12,24	100 base pair ladder
		22	Negative control

Anal swabs were typically processed in batches of 35. Depending on RNA concentration, RT-PCR was performed on average between 25 and 30 samples. These samples were subsequently amplified by PCR and ran on agarose gels as above.

7.5.4 SOUTHERN TRANSFER

Southern transfer of PCR gels that showed bands corresponding to the base pair size of both IF-γ and IL-4 had no corresponding bands when probed by the Southern

transfer method. Positive and negative controls were intact. It was concluded after this analysis that there was either no anal sample cytokine mRNA present, or it was present in very low concentration and beyond the sensitivity of the experimental method used.

7.6 DISCUSSION

There is increasing interest in the immunopathogenesis of various diseases within gastrointestinal tissue.(389) Consequently, there has been a need to develop assays to quantify immunological parameters such as cytokine gene expression in these compartments. This is particularly relevant to the field of HIV mucosal pathogenesis as the gastrointestinal epithelium is the site in Western societies where HIV transmission most frequently occurs and where HIV infection is established. It is critical to understand these early events, particulary as the prevention strategy for HIV expands to include mucosal immune responses to preventative or therapeutic vaccination and the local immune response to candidate rectal microbicides. Within this context, and due to the amost ubiquitous presence of sexually transmitted HPV in HIV-positive MSM, it is vital to understand the local influence both of this infection and of HPV associated anal dysplasia.

To date, most studies have used tisssue samples derived from surgical resection specimens or endoscopic biopsies to describe cytokine responses in gut. However it would be clearly advantageous to develop assays based on less invasive procedures such as the collection of anal swabs. As discussed earlier this approach has been used to characterise cytokine gene expression using endocervical brush sampling.(359) Unfortunately in the above study this did not prove possible for the anal canal.

Reasons for this failure to detect cytokine gene expression may be due to a number of different reasons that are discussed below.

7.6.1 CYTOKINE DETECTION BY RT-PCR FROM ANAL CYTOLOGY SWAB SAMPLES

In the above study anal canal samples were taken and immediately placed in denaturing solution and stored at -80 °C. RNA was subsequently extracted by the phenol-chloroform method, quantified and reverse transcribed. PCR was then undertaken with primers specific for GAP-DH, IF-γ, IL-2, IL-4 and IL-10. Lastly, Southern transfer was performed and did not confirm the presence of any cytokine signal. Potential factors associated with the failure to detect cytokine m-RNA with this methodology are discussed below.

7.6.1.1 ANAL SWABS

7.6.1.1.1 CELLULARITY

Anal swabs are hypocellular compared to both cervical cytology material that has at least 2000-3000 squamous cells, and anal biopsies that contain approximately 1 million cells when obtained using endoscopic forceps (J Elliott - personal communication). In this study the subjects had previous anal cytology and a further anal swab taken for HPV detection making it likely that the third study swab taken for the cytokine study contained decreased amounts of cellular material.

The cells present in anal cytology specimens are predominantly squamous cells, with T lymphocytes present in much reduced numbers. While epithelial cells are capable of producing cytokines, Th cells are more likely to secrete both Th1 and Th2 cytokine

mRNA. Additionally, most cytokine secreting Th cells are predominantly found in the sub-epithelial compartment that is not accessible to cytological sampling.

Thus, using the above sampling technique in the anal canal there was likely to be a low starting cell count with cells that were present less likely than PBMCs to secrete cytokines.

7.6.1.1.2 COMPARISON OF ANAL AND CERVICAL SWABS

The previously demonstrated detection of cervical canal cytokines by Scott *et al* using similar methodology to that used above was encouraging although the cervical and anal canals have significant differences from a sampling perspective.(359) Cervical samples are obtained using both an endocervical brush in addition to an ectocervical spatula, wheras the anal samples were taken using only a water moistened Dacron swab because of issues related to subject acceptability. By using the bimodal cervical sampling technique, and because of limited cervical subjective sensation, the ability of clinicians to be more vigorous when sampling the cervix may in part explain the cervical hypercellularity compared to the anal canal. Additionally the anal canal is subject to abrasion with the passage of stool that may remove surface cells that is not an issue in the cervical canal.

Furthermore, the cervix has been shown to be immunologically active with lymphocyte shedding detected in almost 40% of HIV-positive women in one study.(390) This may also be the case in HIV-negative women and so have had an impact on the ability of Scott *et al* to detect cytokines in samples from cervical swabs.

7.6.1.1.3 FAECAL CONTAMINATION OF SWABS

The reduced RNA purity was likely due to the presence of protein in the sample and related to faecal contamination of the anal swab specimens. Anal specimens were rarely grossly faecally contaminated, despite their nature, so discarding swabs based on visual appearance would not have obviated this concern.

Normal faecal volume is partially comprised of gastrointestinal cellular material. It is likely that total anal swab RNA was derived from both anal epithelial cells and other cellular material such as sloughed gastrointestinal luminal cells and may explain the robust signals demonstrated for GAP-DH so giving false reassurance as to the adequacy of the experimental methods.

7.6.1.2 RNA

7.6.1.2.1 RNA DEGRADATION

While every effort was made to reduce the possibility of RNA degradation, it is possible that cytokine RNA degradation may have occurred prior to reverse transcription to cDNA. The management of anal samples following collection was similar to that performed by Dr. Mark Scott with cervical specimens. It is possible that RNAse exists in the anorectum that is not present in the cervical canal, or that the degradation of the likely smaller concentrations of cytokine mRNA was enough to limit its detection when compared to a housekeeping gene such as GAP-DH.

7.6.1.2.2 RNA PURITY

Phenol:chloroform RNA extraction provided less than optimal RNA quality based on spectrophotometric analysis of 260/280 nm light absorption. This problem was addressed by performing repeated rounds of phenol:chloroform extraction that had

little or no effect on quality. In addition, great care was taken to avoid any cellular debris at the phenol:chloroform interface when processing the specimens as capturing the interface would also increase protein content of samples. The poor quality of the RNA was likely again related to the nature of the specimens and in particular protein contamination as discussed above. The use of other RNA isolation methods such as commercially available kits (e.g. RNAeasy.(Quiagen Inc., Valencia, CA, USA)), may have yielded a better quality product, although all isolation systems are highly user dependent.

7.6.1.2.3 RNA QUANTITY

Using the phenol:chloroform method mostly modest amounts of RNA were extracted, however these amounts were sufficient to allow RT-PCR within the volume and concentration limits of the experimental method described above. Repeated rounds of RNA extraction did not alter the amounts of RNA extracted. It should be noted that a typical RNA yield is approximately 1-2% of all RNA in the sample, thus only considering RNA samples of higher quantity (e.g. a 260 nm reading greater than 0.1), or increased specimen purity with a 260/260 ratio over 1.8 for RT-PCR may have yielded an increased chance of cytokine detection.

7.6.1.3 PCR

7.6.1.3.1 PCR CYCLING PARAMETERS

PCR cycling parameters were optimised for several variables including cycle number and Taq concentration for GAP-DH, and each primer pair had optimum Magnesium concentration established. In addition, the PCR machine used was shown to consistently amplify DNA in each individual well.

Due to the presence of highly visible positive control bands on agarose gel electrophoresis using an empirical PCR protocol it was not considered necessary to establish individual denaturing, annealing and extension temperatures for each primer pair. Additionally, using the above method RT-PCR was capable of detecting 2 ng of target mRNA with visualisation of PCR product on an agarose gel for GAP-DH.

Despite this optimisation of PCR cycling parameters no verifiable cytokine mRNA was detectable. Reasons for this likely included low starting concentration of cytokine cDNA after RT, and the presence of PCR inhibitors. Increasing the number of PCR cycles was discounted as a means of increasing detectable PCR product as it was considered that this would also increase the already frequent accessory bands visible on the agarose gels and make specific band detection more difficult. This problem would, however, have been abrogated if initial experimental design had included plans to perform a Southern transfer on each specimen. The use of quantitative real-time PCR may have resolved the issue with low starting mRNA concentrations as is discussed above.

7.6.2 ALTERNATE METHODOLOGY FOR CYTOKINE DETECTION IN THE GASTROINTESTINAL TRACT AND GENITAL TRACT

A number of alternate methodologies exist to increase cytokine m-RNA detection and are outlined above. Although the initial intent of the experiment was to demonstrate cytokine gene expression in material collected by minimally invasive anal swabs, the most effective change in experimental design may have been to transfer the sampling technology from anal swab cytology to anal tissue biopsy collection. This change would have increased cell yield, captured the cell rich submucosa, and if successful allowed the investigator to assess cytokine gene expression in tissues with both normal and dysplastic appearances using high-resolution anoscopy.

Tissue biopsy has been used to obtain samples from the cervix with subsequent detection of cytokines as described above.(361) (333) (391) This methodology is also used at the University of California Los Angeles (UCLA) Mucosal Immunology Core of the UCLA AIDS Instuitute to detect cytokines from rectal and colonic samples taken at colonoscopy or flexible sigmoidoscopy.(392) UCLA investigators have published data on cytokine gene expression by Real Time RT-PCR in rectal and colonic biopsies in HIV-negative and HIV-positive individuals that qualifies the methodology and has also demonstrated quantitative differences in cytokine gene expression in both gastrointestinal sites, and also in individuals with treated and untreated HIV disease.(392) Preliminary data has more recently also demonstrated the feasibility of detecting cytokines using the same methodology from anal biopsy material (I. McGowan - personal communication).

7.6.2.1 QUANTITATIVE REAL TIME PCR

Real-time polymerase chain reaction, also called quantitative real time polymerase chain reaction (QRT-PCR) or kinetic polymerase chain reaction, is a laboratiory technique used to simultaneously quantify and amplify a specific part of a given DNA molecule. It is used to determine whether or not a specific DNA sequence is present in the sample; and if it is present, what the numbers of copies in the sample are. The procedure follows the general pattern of polymerase chain reaction, but DNA is quantified after each round of amplification; this is the "real-time" aspect. Two common methods of quantification are the use of fluorescent dyes that intercalate with double-stranded DNA, and modified DNA oligonucleotide probes that fluoresce when hybridised with a complementary DNA. While this technology was not available in

the Palefsky lab, its use combined with RT may have allowed quantification of low abundance cytokine mRNA at higher cycle numbers.

7.6.2.2 ENZYME LINKED IMMUNOSORBENT ASSAY

Investigators in the female genital tract have used cervical cytology to detect cytokines but instead of RT-PCR have used an enzyme-linked immunosorbent assay (ELISA).(393) These investigators demonstrated that levels of Th1-cytokines IFN-γ and TNF-α were negatively correlated with Th2-cytokines IL-6 and IL-10 in HPV-positive normal and LSIL subjects. No correlation was observed for subjects with HSIL.

7.6.2.3 IMMUNOASSAY

As an alternative to the use of biopsy tissue, Lettesjo *et al* have demonstrated the presence of cytokines in stool from subjects with irritable bowel syndrome (IBS) and collagenous colitis (CC) using a method that used immunoassays of faecal fluid homogenate supernatant.(394) However, in these samples only 2 of 11 CC subjects had detectable Interleukin 1 beta, and no IBS or CC subject had detectable tumour necrosis factor alpha. This may indicate, similar to the methodology presented above, that only small and mostly undetectable amounts of cytokine are present.

7.6.2.4 IN-SITU HYBRIDISATION (ISH)

Inflammatory bowel disease (IBD) encompasses Crohns Disease that typically displays a Th1 type response and Ulcerative Colitis that typically displays a Th2 type response. Investigators have shown that cytokines may be detected by ISH of both gastric and rectal tissue in patients with UC and confirmed the predominantly Th2 type response. (395)

7.6.2.5 LUMINEX™

With over 100 cytokines/chemokines of clinical interest, investigators need to analyse each sample for multiple cytokines order to correlate a specific disease process with changes in cytokine levels. Using traditional methodologies, limited sample sizes or budget restrictions can often prohibit this kind of testing. Luminex™ technology offers rapid and sensitive testing of multiplexed cytokine measurements with sensitivity comparable to traditional ELISA-based systems but with smaller sample size requirements.(396) This methodology has been used with success in situations where gastrointestinal tissue explants have been maintained in the laboratory and functional assays performed on fluids supernantants.

7.6.3 ANORECTAL CO-INFECTION WITH STI

No testing for sexually transmitted infection (STI) was made prior to anal swab collection, neither was anal symptoms noted. While it is likely that any STI/inflammation would increase cytokine gene expression, this omission was of academic interest only in the context of absent cytokine detection. However, of interest Scott *et al* has recently published data that showed increased levels of IF-γ, IL-10, and IL-12 by RT-PCR of specimens collected by cervical swabs in women with current *C. trachomatis* infection. (397)

7.6.4 AIM 2

As the RT-PCR for GAP-DH was almost always positive, and considering the strong signals detected for each positive control it is unlikely that the RT-PCR reactions were inadequate, and more likely that cytokine mRNA was not present in detectable quantity using the above technique. Thus, it was not possible to progress with Aim 2.

7.7 FUTURE CONSIDERATIONS

The anal canal has largely been ignored scientifically as a potentially immunologically active site. More recently, and in the context of HPV associated disease, statistics indicate that HIV-positive individuals have an increasing incidence of anal cancer. Empirical translation of cervical cytological screening techniques are being used to detect these potentially precancerous lesions but very little information has been presented addressing the immunological impact of HPV and the development of anal dysplasia. The above study failed to demonstrate the presence of cytokines using the methodology described, however, as discussed above the most appropriate change in methodology would have been to use biopsy tissue instead of exfoliative cytology material. This would have likely abrogated issues related to hypocellularity and faecal contamination, and additionally by using technologies such as real-time PCR may have allowed manipulation of cycling parameters in response to cytokine signal.

Ultimately despite the considerable challenges involved, the investigation of the anal canal remains vital to both inform the pathogenesis of HPV associated anal dysplasia and also to provide methods of assessing anal immune signal in the context of HIV infection prevention strategies such as anorectal microbicide development.

CHAPTER 8

THE PROJECT 'EXPLORE' ANAL HUMAN PAPILLOMAVIRUS/ANAL CYTOLOGY SUB-STUDY

8.1 INTRODUCTION

What follows is a description of the development, set-up and initiation of a US multi-site clinical study. An abridged study protocol is presented, and study results published by Chin-Hong *et al* briefly presented and discussed.

8.2 PROJECT EXPLORE

Project EXPLORE (also known as HIV-NET 015) was a randomised controlled Phase IIb trial of a behavioral intervention to prevent HIV infection in a cohort of 4295 HIV-negative MSM funded by the National Institute of Allergy and Infectious Disease (NIAID) Division of AIDS, HIV Network. The study was conducted in six US metropolitan areas (New York City, San Francisco, Denver, Seattle, Boston and Chicago) and employed counseling sessions and STD testing over a 3-year period. The intervention arm received ten counseling sessions followed by maintenance sessions every 3 months while the standard of care arm received twice-yearly counseling. The design of the study allowed enrollment of ethnically and socio-economically diverse HIV-negative MSM with a wide age range in US urban centers. The assessments over a three-year period provided HIV incidence and prevalence data, in addition to demographic, behavioural, medical (sexually transmitted infection) data at each 3-6 month study visit. The published report for EXPLORE can be found in Appendix 2.

8.3 HPV SUB-STUDY

The HPV sub-study to EXPLORE was conceived and subsequently implemented at UCSF with the goal of detecting the prevalence and incidence of HPV and anal cytological abnormality in a population of HIV-negative MSM, in addition to assessing the relationship of these parameters to HIV incidence.

8.3.1 BACKGROUND

The anal neoplasia study (ANS) at UCSF is the largest natural history study of anal dysplasia in HIV-positive and -negative MSM in the US. Most of the ANS subjects were initially recruited from the San Francisco Men's Health Study (SFMHS) - a prospective epidemiological study designed to learn more about the natural history of HIV/AIDS that was funded by the NIAID in 1983. Men 25-54 years old were recruited then interviewed and tested for HIV infection at six-month intervals. To date this study has provided invaluable information on sexual risk behaviour and HIV incidence in this population.

The men subsequently recruited to the ANS were already at least 35 years old if drawn from the first wave of SFMHS participants, and most other participants were aged from their mid-thirties to mid-forties at study entry. This older population had likely already been exposed to and acquired anal HPV and subsequently developed anal cytological abnormalities. Thus, the study was unable to fully address incident anal HPV and anal cytological abnormalities particularly in an HIV-negative population as the study predominantly recruited HIV-positive men. The study also had too few elderly men to adequately address the effects of age on anal HPV and anal cytological abnormalites.

The EXPLORE study provided a unique opportunity to address age-related anal HPV and anal cytological abnormality incidence, prevalence and the effect of these parameters on HIV seroincidence in an HIV-negative population of MSM.

The evolution of the HPV sub-study study to EXPLORE may be divided into three parts; Protocol Development and Site-Preparedness, Regulatory Considerations and Communication Strategy as discussed below.

8.3.2 PROTOCOL

8.3.2.1 PROTOCOL TEAM

The protocol was developed by Dr. Cranston and Dr. Palefsky with consultation and input from Dr. Darragh (anal cytopathologist) and Ms. Da Costa (Palefsky laboratory manager), and latterly the four sub-study site PIs.

8.3.2.2 PROTOCOL DEVELOPMENT

The protocol was divided into sections devoted to Background, Objectives, Study Design, Participant Eligibility, Study Procedures, Laboratory Specimens, Evaluation of Outcomes, Data Collection, Statistical Considerations, Human Subjects, and Administrative Procedures.

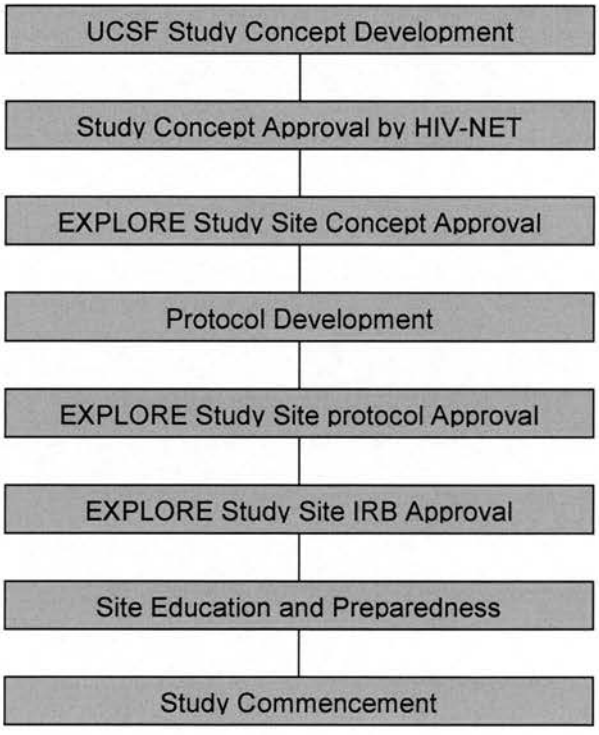
Dr. Cranston wrote initial drafts of each section that were then modified after review by Dr. Palefsky, with the exception of the Statistical Considerations section that was authored by Dr. Eric Vittinghoff, the biostatistician for the EXPLORE study.

Dr. Teresa Darragh (cytopathologist) and Ms. Maria DaCosta (laboratory manager) were involved in discussions at the study concept and protocol development stage to allow for forward planning of time/personnel management that facilitated processing the specimens at UCSF. This was particularly important for anal cytology as this influenced results reporting and time to clinical care for participants with abnormal

anal cytology results. HPV specimen results were not made available to study participants, as anal HPV testing is neither FDA approved nor standard of care and were stored and batch tested according to laboratory personnel availability.

The final protocol appears in Appendix 3 and an abridged form of this protocol appears below in section 8.2. The flow chart representing the study process is shown in Figure 8.1.

Figure 8.1: The HPV sub-study development process



8.3.2.3 SITE PREPAREDNESS

8.3.2.3.1 STUDY SITES

Following HIV-NET approval of the study concept, the six EXPLORE study sites were contacted and asked to consider taking part in the HPV sub-study. Four sites and

PIs were willing to participate; San Franscisco CA (Grant Colfax), New York NY (Beryl Koblin), Denver CO (Franklyn Judson), and Boston MA (Kenneth Mayer). Two sites (Seattle and Chicago) declined to take part in the study as they did not consider it would be feasible to implement the study due to the demands of EXPLORE.

8.3.2.3.2 TRAINING MATERIALS

The HPV sub-study had a limited budget that did not include travel, and thus no on-site training of study investigators or clinical staff was possible (with the exception of the San Francisco site training that was undertaken by Dr. Cranston). Thus, most training was accomplished remotely by creating video presentations of the anal cytology testing method and common counseling messages relevant to each possible anal cytology diagnosis (e.g. ASC-US, LSIL, HSIL).

Dr. Cranston scripted, set up filming with the assistance of the media department at UCSF, and appeared in both videos entitled 'How to take an anal cytology specimen', and 'Counseling messages for providers and study subjects regarding anal cytology and HPV testing'. Scripts for both video presentations appear in Appendix 4 and Appendix 5.

8.3.2.3.3 SUPPLIES

Dr. Cranston supplied all study sites with Cytyc™ ThinPrep anal cytology vials, Dacron™ swabs, packaging and labeling materials that were appropriate for safely transporting biological specimens by air according to Federal Airline Association guidelines, and set up a dedicated shipping account.

8.3.2.3.4 REFERRAL RESOURCES FOR ABNORMAL ANAL CYTOLOGY RESULTS

Prior to study initiation Dr. Cranston provided all sites with the names and contact information of local medical providers that were appropriate referral resources for subjects with abnormal anal cytology.

8.3.2.3.5 REAL-TIME SITE PROBLEM RESOLUTION

At study commencement Dr. Cranston provided a 'hot-line' pager for any urgent real-time clinical care questions that arose relating to participant questions on HPV or anal cytology interpretation or counseling, or any other pressing clinical site issue. This allowed problem resolution while the participant was in the clinic and did not require additional unscheduled return visits or impact clinic staff time.

8.3.3 CLINICAL TRIAL REGULATION

8.3.3.1 INSTITUTIONAL REVIEW BOARD (IRB)

Most large healthcare institutions in the US have an institutional review board/independent ethics committee (IRB/IEC) whose membership is drawn from a pool of individuals working in the same institution and community representatives. This group is formally designated to review and monitor biomedical and behavioural research involving human subjects that historically were initially developed in direct response to research abuses earlier in the twentieth century. In accordance with FDA and Health and Human Services (HHS) regulations, an IRB has the authority to approve, require modifications in (to secure approval), or disapprove research. An IRB performs critical reulatory oversight functions to ensure that research conducted on human subjects is ethical. In the US, IRBs are mandated by the Research Act of

1974, and are themselves regulated by the Office for Human Research Protections (OHRP) within HHS.

As UCSF was not a study site, no IRB documentation was submitted to this site and it was the responsibility of the site PI to submit the IRB request as an ammendment to the EXPLORE protocol. Issues that were relevant to the IRB application that formed an integral part of the protocol related to the Human Subjects section was concerned with; risk of taking an anal cytology specimen, impact of an abnormal anal cytology diagnosis both psychologically and in terms subsequent medical referral, and protection of confidential medical information. At all clinical sites the IRB ammendments were approved without difficulty.

8.3.3.2 EVOLUTION OF CLINICAL TRIAL REGULATION

The current regulatory environment in the US has evolved considerably since the HPV sub-study was conceived and implemented. All studies now require Health Insurance Portability and Accountability Act (HIPAA) documentation that ensures the security and privacy of health data. Certificates of Confidentiality are required to limit the availability of sensitive personal information such as illicit drug use/sexuality to external investigators such as the Federal Government. Studies may additionally be subject to external audit review during the study by the Federal funding body. While all these regulations have been implemented to safeguard the wellbeing of the study participant and their personal information they have significant consequences for study costs and time to study implementation.

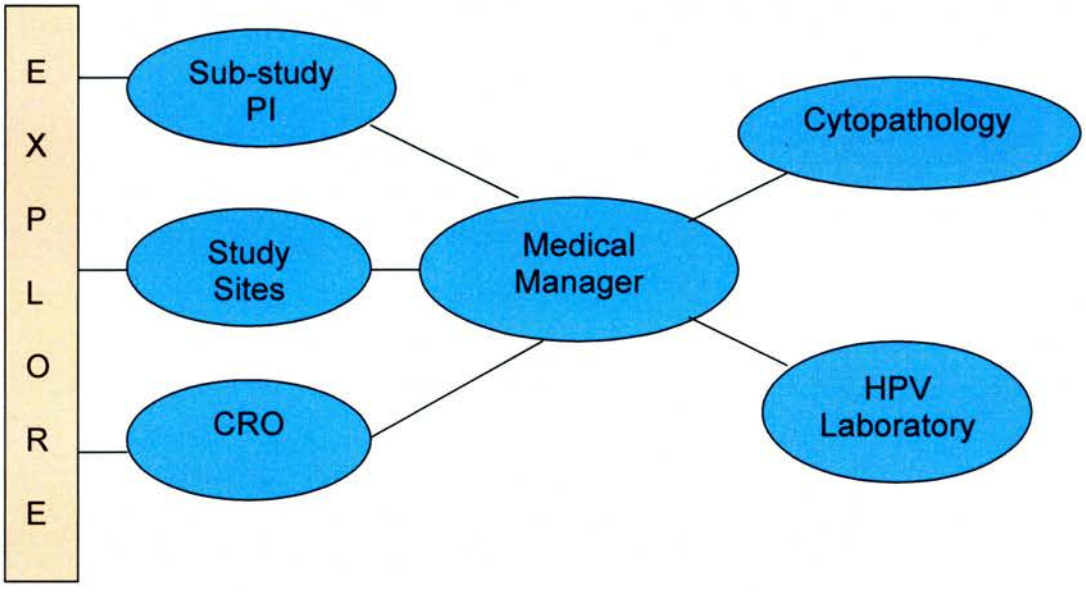
8.3.4 COMMUNICATION STRATEGY

8.3.4.1 CONTRACT RESEARCH ORGANISATION (CRO)

Abt Associates Inc. (Cambridge, MA) functioned as the CRO with specific remit for the HPV sub-study in addition to their responsibilities as CRO to EXPLORE. This for-profit company applies research and consulting techniques to a wide range of issues in social, economic, and health policy; international development; business research and consulting; and clinical trials and registries. One of the strengths of this company is that they employ experts in the specific field of research that in this case included persons with extensive experience in the field of HIV/AIDS both for EXPLORE and the HPV sub-study.

Abt Associates were responsible for suggesting that the initialisation of the sub-study be delayed until after the initiation of EXPLORE due to the intensity of EXPLORE set-up, and the use of study site resources for recruitment and retention. Thus the HPV-sub-study initiation, with the full support of site PIs, was delayed approximately 8-12 months. Subsequently, after initiation of the parent study the first sub-study subjects were recruited at the month 12 EXPLORE visit and offered study entry at each 6-month visit subsequently. This delay had clear implications for recruitment and in particular the number of longitudinal data points for subjects entering the sub-study at later visits for incidence analysis of HPV, anal cytological abnormalities and HIV infection.

Figure 8.2: Organogram of the EXPLORE HPV sub-study



8.3.4.2 TELECONFERENCES

Teleconferences were the mainstay of study communication. Initial conference calls were designated every four weeks when the study was being set up, and after the hiatus due to EXPLORE start-up prior to study initiation. The conference calls were coordinated by Abt Associates, led by Dr. Cranston and Dr. Palefsky and attended by each PI or designated study site personnel. Initial calls were concerned with the protocol, IRB applications and timelines around this process. Later conference calls concentrated on issues of training study-site personnel to obtain anal cytology samples, ability of study site personnel to counsel subjects on normal or abnormal anal cytology results, adequacy of supplies to conduct the study and ensuring that specimens were shipped according to study protocol.

8.3.4.3 STUDY DOCUMENTATION

Due to the length of the start-up period and complexity of maintaining four clinical sites all study-associated material was collated and kept at UCSF. This included the final protocol, regulatory information, teleconference action items, Federal shipping regulations for biological specimens, and contact information for the FedEx account, vendors of study supplies, all study sites PI's and associated personnel, and abnormal anal cytology referral sources. This, along with a short period of overlap between Dr. Cranston and Dr. Chin-Hong facilitated smooth transition of the medical management of this multi-site study.

8.3.5 STUDY COMMENCEMENT

Dr. Cranston was present at UCSF for the first few weeks of study enrollment prior to transferring responsibility to Dr. Chin-Hong.

8.3.6 ANAL CYTOLOGY ADEQUACY

This was the first time that many study-site personnel had taken an anal cytology specimen and there was concern as to specimen quality. Dr. Darragh was asked to perform rapid quality control on the initial 20 specimens from each site in order to provide timely feedback to personnel.

8.4 THE ABRIDGED EXPLORE HPV SUB-STUDY PROTOCOL

HPV infection is one of the most common sexually transmitted agents among both men and women, and HPV-associated anal condyloma are one of the most common sexually transmitted diseases among MSM practicing receptive anal intercourse. HPV has received much attention in the last decade due to its causal association with

anogenital squamous cell cancer, including cancer of the cervix, vulva, vagina, penis and anus.

There are two important reasons to study anal HPV infection and HPV-related lesions in the anal canal of MSM. The first is that anal HPV infection may lead to development of anal cancer and its putative precursors, ASIL. An understanding of the biology of HPV infection and ASIL is critical to the development of an effective strategy for prevention of potentially preventable anal cancer in MSM. The second reason is perhaps even more important, which is that anal HPV infection and ASIL may represent important risk factors for the acquisition of HIV infection among HIV-negative men, as well as new strains of HIV among men who are already HIV-positive.

8.4.1 ANAL HPV INFECTION AND ASIL AS RISK FACTORS FOR ANAL CANCER

The primary reason to expand our knowledge about anal HPV infection and ASIL is their putative relationship with invasive anal cancer. Historically, most anal cancers in men and women occur after the age of 50 years but anal cancer has recently been increasing among younger men. The relationship between anal cancer incidence and the HIV epidemic is not yet clear. One study suggested that the increase in anal cancer among single, never-married men in San Francisco between the ages of 25 and 44 years predated the HIV epidemic.(267) In contrast, others have shown that the rate of invasive anal cancer increased with increasing proximity to an AIDS diagnosis, suggesting an association between anal cancer and HIV-related immunosuppression.(276)

Since development of cervical cancer is preventable through detection and treatment of CSIL before it progresses to cancer, it is highly likely that treatment of ASIL will

prevent development of anal cancer. Anal cancer is thus one of the few preventable malignancies in either HIV-positive or HIV-negative men, and an understanding of the natural history of ASIL and associated risk factors is clearly needed to develop appropriate prevention strategies.

For the last five years, the UCSF ANS has performed studies on the prevalence of anal HPV infection and ASIL in a cohort of 346 HIV-positive and 242 HIV-negative men, as well as changes in infection and disease over a 4-year follow-up period.(102, 206) The subjects were enrolled primarily from the SFMHS and the mean age at enrollment of the HIV-positive men was 42 years (range, 24-64). The mean age of the HIV-negative men was 45 years (range, 26-73). A high proportion of HIV-positive and HIV-negative men had anal HPV infection and ASIL at baseline and after 4 years of follow-up; the projected incidence of HSIL was 49% among HIV-positive men and 17% among HIV-negative men. These data indicate that a significant proportion of both HIV-positive and HIV-negative men are at risk of developing anal cancer.

This EXPLORE sub-study study was performed in HIV-negative men, but its results have important implications for HIV-positive men. Since a high proportion of HIV-negative men had anal HPV infection at baseline, we believe that many HIV-positive men will have acquired HPV infection and possibly ASIL prior to acquisition of HIV infection. An understanding of anal HPV infection and ASIL among HIV-positive men is therefore dependent on understanding the dynamics of infection and disease prior to seroconversion.

Risk factors for acquisition of HPV infection and development of ASIL also remain poorly understood. In the UCSF cohort study described above, we did not have a sufficient number of HIV-negative men to perform multivariate analyses of risk factors. Even more importantly, since most of the men were in their forties, we

believe that they may have acquired HPV infection too long ago to provide meaningful data. For this reason, studies of HIV-negative men younger than age 26 provides an ideal opportunity to better understand risk factors for HPV infection and ASIL. To illustrate this point, we have been studying anal HPV infection in high-risk HIV-positive and HIV-negative women, mostly age 35 or older. When queried about receptive anal intercourse as a risk factor for HPV infection and ASIL, there was no clear association. However, when we repeated this study among adolescent women who had recently initiated sexual activity, we found anal HPV infection in nearly 30%, and there was a clear correlation with receptive anal intercourse in this group.

8.4.2 ANAL HPV INFECTION AND ASIL AS RISK FACTORS FOR HIV SEROCONVERSION

Several studies have documented the role of sexually transmitted diseases (STD) as cofactors for HIV acquisition.(398-401) To date there have not been any rigorous studies on the role of HPV infection or ASIL in potentiating HIV acquisition. The role that STDs play in HIV acquisition may be twofold. Firstly, STDs are often associated with mucosal friability and the increased possibility of body fluid exchange, including blood, may enhance HIV transmission. Men who have internal anal lesions, whether low-grade or high-grade, bleed very easily when a swab is passed into the anal canal to obtain specimens for HPV testing or cytology. Insertion of objects into the anal canal, as in receptive anal intercourse, may similarly lead to more frequent and larger volumes of bleeding if ASIL is present. Secondly, STDs may lead to inflammatory responses, and the increased presence of lymphocytes prone to HIV infection may also potentiate HIV acquisition. HPV-related diseases may lead to HIV acquisition for both of these reasons. Biopsies of ASIL frequently show a lymphocytic infiltration, similar to cervical SIL lesions in which the majority of the lymphocytes in cervical

lesions are CD4 cells. Therefore it is likely that cells susceptible to HIV infection are present in larger quantities in ASIL lesions, at sites that are also likelier to bleed. For both these reasons, we believe that HPV infection and ASIL increase the risk of HIV acquisition among HIV-negative men.

Additionally, HIV-positive men who have ASIL at the time of engaging in receptive anal intercourse may be at risk of exposure to new HIV strains and may also be likelier to transmit HIV to active partners. Since women have been shown to acquire cervical HPV infection soon after initiation of sexual activity, we hypothesise that MSM also acquire HPV infection soon after initiating receptive anal intercourse. If so, then they may have HPV infection and possibly ASIL at a time of their lives when they may be most likely to practice unsafe sex with a large number of partners. If data do show that ASIL is a risk factor for HIV acquisition, this may have important implications for development of new strategies to prevent HIV transmission. Specifically, detection and treatment of ASIL in high-risk HIV-negative MSM may be useful to lower the risk of HIV acquisition.

In summary, more information is needed on the biology of anal HPV infection and ASIL among MSM. These data are needed to better understand HPV infection and ASIL as risk factors for anal cancer in both HIV-negative and HIV-positive men. They are also needed to determine if HPV infection and ASIL are risk factors for HIV acquisition.

8.4.3 STUDY RATIONALE

Anal cancer is a relatively common malignancy among MSM and may be even more common among HIV-positive MSM. Data on anal HPV infection and ASIL from a cohort of 346 HIV-positive and 262 HIV-negative MSM at UCSF show that anal HPV infection and ASIL are very common among men in the 35-45 year age range.(91, 102) Our follow up of the cohort showed that the 4-year projected incidence of the putative anal cancer precursor lesion HSIL was 49% among HIV-positive men and 17% among HIV-negative men. While men with anal HPV infection and ASIL are probably at increased risk of anal cancer, little is known about the prevalence or incidence of HPV infection and ASIL in men at either end of the age spectrum, i.e., men over the age of 50 years and men aged 25 years or less. Although these studies will be performed in HIV-negative men, they will also have important implications for HIV-positive men, since we believe that many HIV-positive men will have acquired HPV infection and possibly ASIL prior to acquisition of HIV infection. An understanding of anal HPV infection and ASIL among HIV-positive men is therefore dependent on understanding the dynamics of infection and disease prior to seroconversion. Knowledge of the natural history of anal HPV infection and ASIL in the early years after sexual activity begins will therefore be very important. Like cervical cancer, anal cancer may be preventable through screening for and treatment of ASIL, and data collected in this study could have important implications for development of anal cancer screening programs for both HIV-positive and HIV-negative men.

8.4.4 STUDY OBJECTIVES

The following objectives were addressed:

- To define the prevalence, incidence and risk factors for anal human papillomavirus infection in a cohort of sexually active HIV-negative MSM.
- To define the prevalence, incidence and risk factors for anal cytologic abnormalities in a cohort of sexually active HIV-negative MSM.
- To determine the association between HIV seroconversion and detection of anal cytologic abnormalities or anal HPV infection in a cohort of sexually active HIV-negative MSM.

8.4.5 STUDY DESIGN

The study characterised HPV infection and anal cytologic abnormalities in approximately 1300 MSM chosen from among 2900 (four out of six sites) men recruited into EXPLORE. All eligible participants were offered enrollment over a maximum of a two-year accrual period until a total of 1400 - 2000 men were enrolled to July 31, 2002.

EXPLORE participants were offered enrollment in the HPV study at the Month 12 study visit, unless the participant had been on study more than 486 days (closing date of the Month 12-visit window) when the study was implemented. Thus some participants were offered enrollment at the Month 18 study visit, and a few were offered enrollment at the Month 24 study visit and Month 30 study visit.

At HPV study enrollment and at three 6-month interval visits (or two 6-month interval visits for Month 30 enrollees) and for the duration of this HPV study, a minimum of one anal swab was obtained from each participant. The swab was used for anal

cytology (per our standard protocol) using the Thinprep method as well as for HPV testing.

8.4.6 PARTICIPANT ELIGIBILITY

This study included men at high-risk for HIV infection enrolled in the EXPLORE: A Randomized Clinical Trial of the Efficacy of a Behavioral Intervention to Prevent Acquisition of HIV Among Men who have Sex with Men.

8.4.6.1 INCLUSION CRITERIA

Persons were included in the study if they met the following criteria

- Enrolled in the EXPLORE
- Willing and able to provide written informed consent.

8.4.6.2 EXCLUSION CRITERIA

Persons were excluded from the study if they:

- Had an obvious psychological/psychiatric disorder that would invalidate the informed consent process, or otherwise contraindicate participation in the study.
- Had any other condition that in the opinion of the study site Principal Investigator will interfere with achieving the study objectives.

8.4.6.3 CONDITIONS FOR WITHDRAWAL

Participants could withdraw from study participation at any time, for any reason, without loss of other benefits or services to which they were entitled. Withdrawal from the HPV ancillary study did not affect participation in the EXPLORE trial. Participants could be withdrawn from the study due to inability to comply with study

procedures. Participants could be withdrawn at the request of the study site investigator, following review with the co-chairs, protocol bio-statistician, and the Protocol Coordination Team (PCT) Project Officer.

8.4.7 STUDY PROCEDURES

An overview of the study visit and procedure schedule is presented in Table 8.1.

Table 8.1: Schedule for visits and procedures during the HPV sub-study.

Study Month – HIVNET 015	12	15	18	21	24	27	30	33	36	39	42
Enrollment in HPV Study	D_1		D_2		D_3		D_4				
Laboratory tests											
Anal Cytology	D_1		D		D		D		D_2 D_3 D_4		D_3 D_4
Anal HPV Testing	D_1		D		D		D		D_2 D_3 D_4		D_3 D_4

D = HPV study visits for all enrolled participants
D_1= HPV study visits for those who enroll at the Month 12 HIVNET 015 visit only
D_2= HPV study visits for those who enroll at the Month 18 HIVNET 015 visit only
D_3= HPV study visits for those who enroll at the Month 24 HIVNET 015 visit only
D_4= HPV study visits for those who enroll at the Month 30 HIVNET 015 visit only

8.4.8 CONSENT

Each participant had to sign a consent form prior to participating in the HPV study.

8.4.9 SPECIMEN COLLECTION

Once the study was discussed with the subject and the consent form signed, anal cytology specimens were collected using the ThinPrep method as previously described above.

8.4.10 FOLLOW-UP OF ANAL CYTOLOGY RESULTS

Anal cytology results were usually made available to subjects within 2 months (and no longer than 4 months after collection) of their visit. If the cytology showed HSIL or cancer, the subject was notified as soon as those results were available.

8.4.11 LABORATORY SPECIMENS

Specimens were collected and sent to the Department of Pathology, UCSF (cytology samples) and the Department of Laboratory Medicine, UCSF (HPV samples).

8.4.11.1 ANAL CYTOLOGY TESTING

Thinprep anal cytologic smears were prepared according to the manufacturer's instructions. Dr. Teresa Darragh, an expert in gynecologic pathology and is one the most experienced pathologists in the world in interpreting anal cytology and histopathology, read each slide. Slides were interpreted independently and without knowledge of the results of anal HPV DNA testing. The criteria for grading anal cytologic smears were identical to those for cervical cytology as discussed above.

8.4.11.2 HPV TESTING PROTOCOLS

HPV testing was performed using PCR, and provided information on the presence of 39 different HPV types. This test detects both high and low level infections. By determining the presence of each type individually, it was possible to analyze other combinations such as HPV 16 and 18 together. We determined how often these individual types persist and whether we could detect new types over time.

To perform PCR, an aliquot of the Cytyc fixative was removed from each specimen when the bottle was opened for the first time. The specimens were heated at 56 ^{o}C for

one hour to inactivate HIV and digested with 10 mg/mL proteinase K. The PCR protocol that we used was adapted from a method by Ting *et al* and has been used by others for many different studies (16). The sample for PCR was added to a tube containing one ml ammonium acetate/EtOH solution, and the DNA allowed to precipitate at –20 ^{o}C overnight or at –70 ^{o}C for one hour. Thirty-five cycles of amplification were performed. Detection of the PCR products was performed using a non-radioactive enhanced chemiluminescence (ECL) dot blot technique. Each membrane contained a set of negative L1 hybridization controls that include human HPV-negative cells and specimens containing all the components of the reaction except target DNA. Positive controls consisted of plasmid amplifications of the different HPV types. Each membrane also contained five specimens chosen at random to be amplified and probed in duplicate, and therefore 5 % of our specimens were tested twice as a check for assay reliability. The PCR product was fixed to the membrane in a UV-crosslinker. ECL detection was performed according to the manufacturer's (Amersham, Piscataway, NJ) instructions, and the blots exposed to X-ray film at room temperature for 15 minutes and two hours. The blots were probed with a generic probe mix consisting of biotinylated HPV L1 PCR products from HPV 11, 16, 18, and 51. A second membrane was probed with ß-globin oligonucleotide probe to detect the presence of human DNA and confirm the integrity of the amplification reaction. Ten more membranes containing specimens positive with the generic probe were made and probed with each of the following types individually: 6, 11, 16, 18, 26, 31, 32, 33, 35, 39, 40, 45, 51, 52, 53, 54, 55, 56, 58, 59, 61, 66, 68, 69, 70, 73, AE2, Pap 155, Pap 291, and the following types together in a probe mixture: HPV 2, 13, 34, 42, 57, 62, 64, 67, 72, W13B. The presence of a dot on the membrane with a probe for a given HPV type indicated positivity for that type. If a specimen was

negative for ß-globin, this implied that the specimen contained an inadequate number of cells or an inhibitor of the amplification reaction. HPV results from these specimens were excluded from analysis.

Specimens that were positive with the generic probe, but negative for the 39 specific types were considered to have an "unknown" HPV type. Because of its high sensitivity, PCR by definition must be performed with great care to avoid the possibility of contamination. We took all possible precautions in handling our samples, including separation of pre- and post-amplification products in different parts of our research building. If any of the above negative and positive controls did not work, the reaction was repeated. The UCSF lab performed a large volume of HPV testing for this study and other studies and to date we have not have problems with contamination.

8.4.12 EVALUATION OF OUTCOMES

The outcomes measured were incidence of anal HPV infection, incidence of anal cytological abnormality, prevalence of anal HPV infection, prevalence of anal cytological abnormality, incidence of HIV infection, demographics of subjects with anal HPV infection/HIV infection/anal cytological abnormality, and retention in the study.

8.5 RESULTS OF THE HPV SUB-STUDY PUBLISHED BY CHIN-HONG ET AL

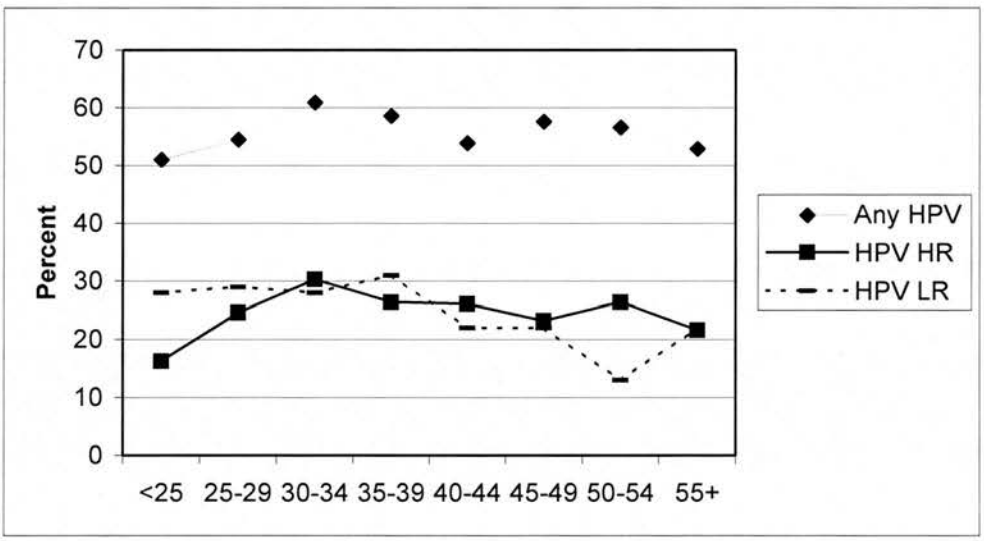
Following Dr Cranston's departure from UCSF the study was handed over to Dr. Peter Chin-Hong who was responsible for its management and subsequent data collection, analysis and publication of results. In the interests of completeness the results presented in the papers and abstract published by Chin-Hong *et al* (Appendices

6, 7, and 8) are included below in addition to a brief disussion of the relevance of this study's findings to the field of anal dysplasia and anal cancer.

8.5.1 PREVALENCE OF ANAL HPV

There is a high prevalence at first study visit of anal HPV of both high-risk and low-risk types through all age ranges studied. (See Figure 8.2) The overall prevalence of anal HPV was 57%, with 26% prevalence of high-risk HPV.

Fig. 8.3: Prevalence of anal HPV DNA detection in men by age group



HPV: human papillomavirus; HPV HR: high-risk human papillomavirus; HPV LR: low-risk human papillomavirus. Figure reproduced courtesy of Chin-Hong (111)

8.5.2 HPV TYPE

HPV-16 was the most commonly identified HPV type. Multiple HPV infections were detected in 45% of participants.

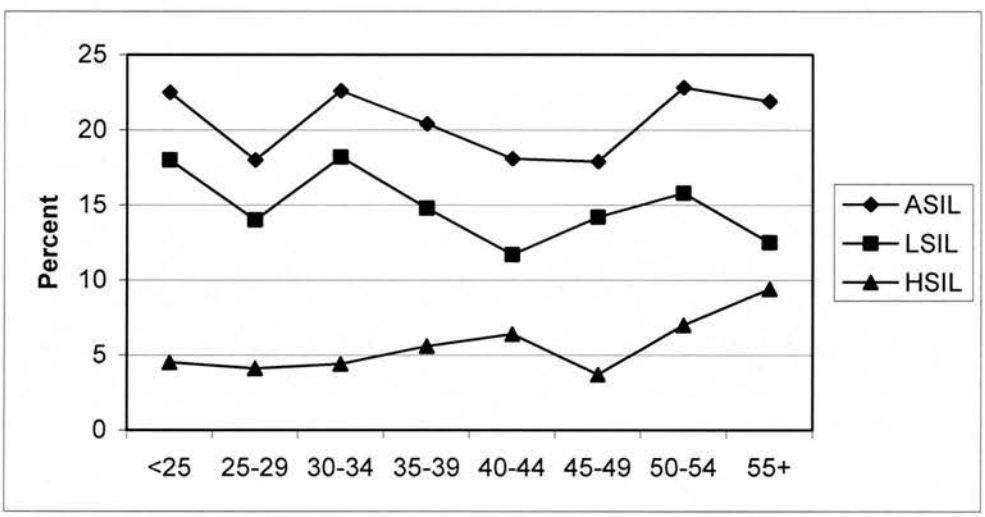
8.5.3 RISK FACTORS FOR ANAL HPV

Risk factors identified for the presence of anal HPV were: history of receptive anal intercourse (Odds ratio (OR), 2.0; P<0.0001), and having more than 5 sex partners within the previous 6 months (OR, 1.5; P<0.0001).

8.5.4 PREVALENCE OF ANAL CYTOLOGICAL ABNORMALITIES

Anal cytological abnormalities were common in all age groups with an average prevalence of 20% (See Figure 8.3)

Figure 8.4: Prevalence of ASIL in men by age group



ASIL: anal squamous intraepithelial lesion; LSIL: low-grade squamous intraepithelial lesion; HSIL: high-grade squamous intraepithelial lesion. Figure reproduced courtesy of Chin-Hong (243)

8.5.5 RISK FACTORS FOR ANAL CYTOLOGICAL ABNORMALITIES

Using multivariate analyses the risk factors for LSIL was associated with having >5 male receptive anal sex partners (P = .03), use of alkyl nitrites in the previous 6 months (OR=1.6 95% confidence interval (CI) = 1.1-2.5; P = .03) or use of injection drugs two or more times per month in the previous 6 months (OR = 19, 95% = 1.3 - 277; P = 0.03), older age at first receptive anal intercourse (P = .004), and infection

with a greater number of HPV types (P = .001 for linear trend). The risk of HSILs was associated with any anal HPV infection (OR 3.2, 95% CI = 1.1 - 9.4; P = .039) and infection with an increasing number of HPV types (P < 0.001 for linear trend).

8.5.6 RISK FACTORS FOR INCIDENT HIV INFECTION

Risk factors for incident HIV infection using univariate analysis showed evidence (P < 0.05) for an association between anal HPV infection (hazard ratio (HR) 2.7, 95% CI 1.0-7.3) and detection of 3 HPV types (HR 3.7, 95% CI 1.4-10.3) in predicting HIV seroconversion and moderate evidence (P < 0.10) for ASCUS (HR 2.4, 95% CI 0.9-6.1). After adjustment for sexual activity, substance use and demographics, there was evidence (P < 0.05) for the effect of 3 HPV types isolated (HR 3.3, 95% CI 1.1-9.9) and ASCUS (HR 2.8, 95% CI 1.1-7.8).

8.6 DISCUSSION OF RESULTS OF THE HPV SUB-STUDY PUBLISHED BY CHIN-HONG ET AL

The epidemiology of cervical HPV infection and cervical cancer precursors has already been established. In the female cervix the prevalence of both HPV infection and cytological abnormality rises sharply in the late teenage years after sexual debut and then decreases over time, with a slight rise in HPV prevalence in the over 55 year old age group.(402) This late peak may be due to new infection related to a new sexual partnership or age related attenuation of immune response and detection of previous latent HPV infection. Cervical HPV infection is most commonly transient with over 90% of infections becoming undetectable by PCR at 2 years.(403, 404) Most cervical infections are with high-risk HPV, particularly HPV 16 and persistence of this and other phenotypically high-risk infections are related to the development of cervical cytological abnormalities. (405) (406)

This study illustrated the epidemiology of anal HPV infection and anal cancer precursors in urban HIV-negative MSM. The epidemiology of HPV infection in the EXPLORE study differed greatly from that described in the cervix. There were high prevalence rates of both high-risk and low-risk HPV throughout all age ranges sampled with no diminution of prevalence over time. Likewise, and again in contrast to the cervix, the prevalence of both high- and low-grade anal dysplasia was constant over all age range samples at 5% and 15 % respectively.

In this population the high rates of both HPV infection and anal cytological abnormality were most likely related to the higher numbers of new sex partners consistently reported in all the age-groups sampled. The median number of new sex partners in the 6 months preceding anal sampling was 8 while, by comparison, sexually active females over age 30 years are much less likely to have similar numbers of new sexual partners.(402, 407)

As there was no increase in anal HPV prevalence over time it is likely that, similar to the cervix, anal infections are also transient with the constant prevalence rates indicating frequent re-infections. Additionally, although HPV type-specific antibodies have been previously shown to develop following HPV infection, the development of protective type-specific immunity is unlikely as the same infection types were detected in both younger and older men, assuming that all men had been exposed to the same HPV types over time.

Although the prevalence of anal dysplasia in this study was 20%, this may be an underestimate based on the limited sensitivity of anal cytological testing with Dacron swabs of 50% for detection of high-grade dysplasia in a population of HIV-negative MSM.(196) Thus, higher numbers of HIV-negative MSM may be at risk for the development of anal cancer.

HPV infection and an atypical squamous cell diagnosis on anal cytology are significantly associated with incident HIV infection. Prevention of anal HPV onfection through vaccination or prompt treatment of HPV-associated lesions may reduce the risk of HIV transmission.

8.7 SUMMARY

8.7.1 HPV SUB-STUDY RESULTS

Although the anal canal and cervix share many embryological, histological and pathological features, particularly in their response to infection with genitotropic HPV infection – there are significant differences in the epidemiology of this infection and subsequent cytological response in the cervical and anal canal of sexually active adults. Likely related to frequent sexual exposure over many decades of life HIV-negative MSM have persistent high prevalence of anal HPV and HPV associated cytological abnormalities. This finding may be a factor contributing to the increased rates of anal cancer in this population compared to the general population. Additionally the presence of HPV and HPV associated anal cytological change may increase risk of HIV infection.

8.7.1 HPV SUB-STUDY SET-UP

The study provides an example of the potential benefits of collaborative research in the context of a multi-site sub-study. Structured and facilitated communication between all groups involved in this type of study at all levels is critical from planning to study execution.

The most significant issue that arose in the initiation of the sub-study was the delay in commencement due to the demands of EXPLORE. However, when the sub-study

commenced, the study-site staff were in a position to both engage participants and to enroll them rapidly. This enthusiastic enrollment allowed final subject numbers to be similar to those projected with the full time allocation. Close liason at all levels from study site to UCSF to the laboratory and cytopathology departments allowed rapid responses to problem as they arose that may have delayed either study enrollment or compromised study samples and data points.

Translational clinical research provides a critical role in bridging basic science to clinical practice. This development has nescessitated increasing regulatory oversight of such studies and whilst this is critical to protect the interests of study participants it may have a significant impact on both study costs and initiation of studies in a timely fashon. The above study was completed prior to federally mandated HIPAA regulation that adds further complexity to the regulatory process.

By definition, translational clinical research requires a multilayered organisation of complementary groups consisting of clinicians, basic scientists, and regulatory groups. This is apparent not only in individual studies but in entire research networks that have evolved to address particular research goals. Only by embracing these challenges in current research can we hope to pursue the goals of the projects while protecting the participants that we ultimately work to serve.

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APPENDIX 1

Self-Collected Versus Clinician-Collected Anal Cytology Specimens to Diagnose Anal Intraepithelial Neoplasia in HIV-Positive Men

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Background: Anal intraepithelial neoplasia (AIN) is common in men who have sex with men (MSM) and may be diagnosed by anal cytology screening.

Methods: One hundred two MSM with clinician-collected anal cytology and histopathology specimens were assessed from a cohort study of AIN at the University of California at San Francisco. The men were given a cytology self-collection kit with written instructions for use and requested to collect the sample 1 month after the clinic visit.

Results: Ninety-one percent of self-collected and 99% of clinician-collected anal cytology samples were adequate for interpretation. The sensitivity of abnormal anal cytology to detect AIN by histology was 68% in self-collected samples and 70% in clinician-collected samples, and the sensitivity to detect AIN 2 or AIN 3 was 71% and 74%, respectively. Cytologic results did not differ by grade between self-collected and clinician-collected samples. Among MSM diagnosed with AIN 2 or 3 by biopsy, 33% of self-collected and 39% of clinician-collected cytology samples were high-grade. The sensitivity of both self-collected and clinician-collected samples to detect AIN 2 or 3 was higher among HIV-positive MSM than among HIV-negative MSM.

Conclusions: MSM with biopsy-proven AIN can self-collect anal cytology samples with sensitivity comparable with that of experienced clinicians. This may facilitate screening for AIN.

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The incidence of anal cancer in the United States is increasing and is 0.8–0.9 case per 100,000 persons among the general population.[1,2] Before the HIV epidemic, the incidence of anal cancer was estimated to be as high as 35 cases per 100,000 persons among men who have sex with men (MSM),[3] with recent data indicating that the incidence among HIV-positive MSM is approximately twice that among HIV-negative MSM.[4,5] Thus, the incidence of anal cancer among both HIV-negative and HIV-positive MSM is higher than the current incidence of cervical cancer among women and is similar to the incidence of cervical cancer before the introduction of cervical cytology screening.[6] Although the magnitude of the increase in anal cancer compared with the general population is greater among HIV-positive MSM than among HIV-positive women, the latter are also at increased risk of anal cancer.[7]

Anal cancer and cervical cancer are similar histologically, and like cervical intraepithelial neoplasia and cervical cancer, anal intraepithelial neoplasia (AIN) and anal cancer are associated with oncogenic human papillomaviruses.[8–12] It is believed that AIN 2 or 3 precedes the development of anal cancer in the same way that cervical intraepithelial neoplasia 2 or 3 precedes cervical cancer (J.M. Palefsky, unpublished observations). Similarly, Bowen disease (perianal AIN 3) may progress to invasive anal cancer.[13,14] Analogous to the treatment of cervical intraepithelial neoplasia 2 or 3 to prevent the development of cervical cancer, treatment of AIN 2 or 3 may prevent progression to anal cancer.

Anal and cervical cytology findings are both described using the Bethesda classification system[15] and are reported, in increasing severity, as atypical squamous cells of undetermined significance, atypical squamous cells suggestive of high-grade change, low-grade squamous intraepithelial lesions, and high-grade squamous intraepithelial lesions (HSIL).[16]

Anal cytology has been studied as a screening test for AIN in MSM.[17–19] In the largest study, using anal histopathology as a gold standard, sensitivity of 69% for HIV-positive individuals and sensitivity of 47% for HIV-negative individu-

als were found using high-resolution anoscopy (HRA) and biopsy to detect AIN.[19] HRA is colposcopy of the anus and perianal region using 3% acetic acid. The sensitivity is comparable with that of cervical cytology screening for the detection of cervical intraepithelial neoplasia in the general female population.[19] The method by which anal cytology samples are processed has also been investigated. In addition to conventional cell fixation and staining by the Papanicolaou method, cytology samples may be prepared using the ThinPrep technique before staining with the Papanicolaou method.[20] Both sampling methods have been validated for the anal canal and found to be comparable in the assessment of anal cytology.[20]

Although somewhat theoretical and not universally accepted, using a state-transition Markov model, we projected that anal cytology screening to identify individuals with HSIL with subsequent treatment of the lesion should be cost-effective to prevent anal cancer when performed annually for HIV-positive MSM and every 2–3 years for HIV-negative MSM.[21,22]

Several barriers currently exist to the implementation of an anal cytology screening program for at-risk individuals. One obstacle is the lack of studies to document that treatment of AIN reduces the incidence of anal cancer. Performance of such studies is hampered by the large numbers of subjects needed, difficulties in assembling an appropriate control group, and the long follow-up needed to assess the impact on cancer incidence. Other obstacles include a paucity of clinicians trained in collecting anal cytology samples, reluctance of some clinicians to address the issue of AIN with their patients, and patients' feelings of embarrassment and fear of potential discomfort associated with the collection of anal specimens. Collection of anal cytology samples by subjects themselves has the potential to obviate some of these concerns.

The purpose of this study was to assess the performance of results from anal cytology samples collected by study participants at home compared with samples collected by experienced research clinicians in a clinic of MSM with a high prevalence of AIN. Anal histopathology was used as the gold standard for this research comparison.

METHODS

Subjects

This study was performed with the approval of the University of California at San Francisco Committee on Human Research. Informed consent was obtained from all subjects. Participants were 505 HIV-positive MSM and 364 HIV-negative MSM enrolled the University of California at San Francisco Anal Neoplasia Study. Of these men, 469 HIV-positive subjects and 256 HIV-negative subjects underwent biopsy at least once during the study. Study participants were examined every 3–6 months with anal cytology and HRA with biopsy of any suspicious lesions. Demographic information was collected and blood samples were obtained for HIV antibody testing.

One hundred twenty-five study participants from our ongoing cohort study of anal neoplasia were invited to participate. Criteria for study eligibility included having had an anal cytology sample taken using glass-slide smears and ethanol fixation and a simultaneous biopsy of any visible anal lesion that appeared dysplastic using HRA. The study participants had previously had anal cytology samples collected in the clinic by their practitioner. The men were consecutively chosen for participation during the study period, and neither cytology nor anal biopsy results were known at the time of enrollment. A clinician provided an explanation of the study, and informed consent was obtained from study subjects. Cytology specimen collection and HRA were performed as described previously with standard glass slides and ethanol fixation used to process the cytology specimens for clinician-collected samples.[19]

Self-Collection of Samples

We previously showed that glass-slide cytology and liquid cytology have similar performance characteristics.[20] The study participants used liquid cytology methods to collect their specimens at home because it was a substantially easier process. The men were given written instructions on how to self-collect an anal cytology sample. The collection kit consisted of 1 bottle of Cytyc ThinPrep sample collection medium (Cytyc Corp., Boxborough, MA), 1 Dacron swab, 2 sealable plastic bags for disposal of sampling materials, and 1 pair of latex gloves. To allow for healing of the initial anal biopsy site, participants were requested to wait 1 month from the time of the original anal cytology and biopsy before collecting their cytology sample.

While wearing latex gloves, the men were instructed to remove the Dacron swab from its sterile package and moisten it with tap water before inserting it 2 inches into the anal canal. Applying gentle pressure to the walls of the anal canal, participants were instructed to remove the swab with a spiral motion over a 10-second period. The swab was to be immediately placed into an open Cytyc bottle containing Cytyc ThinPrep medium and agitated vigorously to disgorge the cells from the swab before it was removed from the bottle. The men were instructed to close the Cytyc bottle tightly, store it at room temperature, and return it to the clinic in a sealed plastic bag within 1 week of specimen collection.

Evaluation of Samples

The same pathologist (T.M.D.) analyzed all clinician-collected cytology and biopsy samples. Because of the different sampling method used, the pathologist knew whether the specimen was clinician collected or self-collected. However, all specimen findings were interpreted without reference to the paired specimen. Cytology findings were classified according

to the Bethesda classification system as inadequate for interpretation (sample contained less than ~2000 to 3000 nucleated squamous cells), negative for squamous intraepithelial lesions, atypical squamous cells of undetermined significance, low-grade squamous intraepithelial lesions, or HSIL.

Statistical Analysis

Statistical analysis was performed using version 8 of the Statistical Analysis System (SAS, Inc., Cary, NC). The sensitivity of anal cytology to detect low-grade squamous intraepithelial lesions and HSIL was defined as the number of specimens with abnormal cytology divided by the number of individuals with AIN. The Fisher exact text was used to assess the level of statistical difference between sample specimens.

RESULTS

MSM who had had a prior clinician-collected anal cytology and HRA examination with anal biopsy were consecutively enrolled from the University of California at San Francisco Anal Neoplasia Study. Approximately 10% of the men invited to participate declined to do so. Of the men who agreed to participate, ~20% did not return the required materials in a timely fashion. Of the 106 remaining participants, 4 were excluded because they had incomplete clinician-collected cytology and clinic biopsy data. One hundred two subjects were therefore included in the analysis, and of these subjects, 82 were HIV positive and 20 were HIV negative. The mean age at the time of sampling was 45 years (range, 29–72 years). Most of the study participants were white non-Hispanic, (n = 89 [87%]); the rest were white Hispanic (n = 2 [2%]), black (n = 3 [3%]), Amerindian (n = 1 [1%]), Asian (n = 3 [3%]), and other (n = 4 [4%]).

Results of the clinician-collected cytology and the corresponding histology are shown in Table 1. Results of the self-collected cytology and the corresponding histology are shown in Table 2. Specimen adequacy was high in both clinician-collected (101/102, 99%) and self-collected (93/102, 91%) cytology specimens, with a significantly higher rate of adequacy seen in the clinician-collected samples ($P = 0.02$). The adequacy rate of self-collected samples was similar for HIV-positive (93%) and HIV-negative (85%) men ($P = 0.37$).

The sensitivity of any grade of anal cytology abnormality for detection of AIN 1, AIN 2, or AIN 3 was comparable between clinician-collected (70%) and self-collected (68%) samples. The sensitivity of any grade of anal cytology abnormality for detection of a high-grade lesion (ie, AIN 2 or 3) was also comparable between clinician-collected (74%) and self-collected (71%) samples. However, the sensitivity of anal cytology to detect AIN in self-collected samples was higher among HIV-positive men than among HIV-negative men. Three (20%) of 15 HIV-negative men with AIN had abnormal cytology compared with 56 (77%) of 73 HIV-positive men ($P < 0.01$). The sensitivity for detection of AIN 2 or 3 specifically in self-collected specimens was also higher among HIV-positive men than among HIV-negative men. Among the 57 HIV-positive men with biopsy-proven AIN 2 or 3, anal cytology was abnormal in 43 (75%), compared with 4 (40%) of 10 HIV-negative men ($P = 0.05$). The sensitivity of anal cytology to detect either AIN overall or AIN 2 or 3 specifically was also higher for HIV-positive men in the clinician-collected samples, and the magnitude of these differences in sensitivity was similar to those seen in self-collected samples ($P = 0.15$ for AIN overall; $P = 1.0$ for AIN 2 or 3). Overall cytologic results did not differ by grade between clinician-collected and self-collected samples. Among men diagnosed with AIN 2 or 3 by biopsy, 39% with AIN 2 and 33% with AIN 3 had HSIL by cytology.

DISCUSSION

Recent guidelines from the US Public Health Service for the treatment of opportunistic infections in HIV-positive individuals indicate that although anal cytology screening has not

TABLE 1. Comparison of Findings for Clinician-Collected Anal Cytology Specimens With Those for Clinic Biopsy

Clinician-Collected Cytology	Anal Histopathology					
	Normal	Atypical	AIN 1	AIN 2 or 3	Insufficient	Total (%)
Normal	4	1	10	18	0	33 (32)
ASCUS	1	0	0	2	0	3 (3)
LSIL	0	1	13	22	0	36 (35)
HSIL	0	0	2	27	0	29 (28)
Insufficient	0	0	1	0	0	1 (1)
Total (%)	5 (5)	2 (2)	26 (26)	69 (68)	0	102

AIN 1 indicates low-grade AIN; AIN 2 or 3, high-grade AIN; ASCUS, atypical squamous cells of undetermined significance; insufficient, insufficient cellular material for interpretation; LSIL, low-grade squamous intraepithelial lesions.

TABLE 2. Comparison of Findings for Self-Collected Anal Cytology With Those for Clinic Biopsy

Self-Collected Cytology	Anal Histopathology					
	Normal	Atypical	AIN 1	AIN 2 or 3	Insufficient	Total (%)
Normal	3	1	8	20	0	32 (31)
ASCUS	0	0	1	3	0	4 (4)
LSIL	0	1	10	22	0	33 (32)
HSIL	0	0	1	23	0	24 (24)
Insufficient	2	0	6	1	0	9 (9)
Total (%)	5 (5)	2 (2)	26 (26)	69 (68)	0	102

AIN 1 indicates low-grade AIN; AIN 2 or 3, high-grade AIN; ASCUS, atypical squamous cells of undetermined significance; insufficient, insufficient cellular material for interpretation; LSIL, low-grade squamous intraepithelial lesions.

yet been formally recommended, anal cytology screening should be considered for HIV-positive men and women.[23] Anal cytology should be used as a screening tool to identify individuals who would benefit from HRA to detect and treat AIN.[19] The current recommendation is to refer patients with any grade of anal cytologic abnormality for HRA.[23,24] Moreover, our group previously showed that detection of HSIL by anal cytology has a high positive predictive value for detection of biopsy-proven high-grade AIN.[19] Thus, inability to confirm high-grade AIN by HRA-directed biopsy in a patient with HSIL by cytology should prompt a repeated examination.

In this study, we compared overall rates of abnormality between samples collected by clinicians and study participants. The results of this study strongly suggest that this population of MSM with previous experience of having had anal swabs used to collect cytology specimens and anal biopsy specimens of lesions clinically suspicious for anal dysplasia obtained by a clinician are capable of self-collecting samples with sensitivity comparable with that of experienced clinicians. This was accomplished with only written instructions on how to self-collect an anal cytology specimen in this experienced population.

The primary goal of anal cancer prevention is to detect and treat high-grade AIN before it progresses to cancer. To this end, we also compared the sensitivity of cytology specimens collected by clinicians and subjects to detect AIN 2 or 3 by biopsy and found that the sensitivity for the 2 groups was comparable. This ability to self-collect anal cytology specimens may allow high-risk populations to be screened outside of a medical setting and allow for subsequent evaluation with HRA and treatment of lesions in individuals with abnormal cytology findings. It could also provide a useful tool for epidemiologic studies of anal cytology and possibly anal human papillomaviruses in large population-based cohorts.

The rates of clinician-collected and self-collected anal cytology specimen adequacy were similar to those seen in a previous study that validated anal cytology as a screening test for AIN.[19] In the present study, specimens collected by clinicians had a slightly lower inadequacy rate than those collected by study subjects. However, this should not affect the role of self-screening because patients with inadequate specimens could be informed of the need to repeat the test. Notably, the sensitivity of anal cytology to detect AIN overall and AIN 2 or 3 specifically was significantly higher among HIV-positive men than among HIV-negative men, a finding consistent with our earlier study.[19] We speculate that this reflects the larger size of lesions typically found in HIV-positive men, leading to a higher likelihood of detection. Another explanation is that HIV-negative men were not as vigorous in obtaining specimens as the HIV-positive men. This is less likely because rates of specimen sufficiency were similar between HIV-positive and HIV-negative men. Further, the clinician-collected specimens were less sensitive for HIV-negative men than for HIV-positive men, similar to our findings for the self-collected specimens. Because participants were enrolled in the study only if they had undergone anal biopsy, the prevalence of AIN in the study was very high, and there were too few participants with normal anoscopic examination findings to assess differences in other measures of screening validity (eg, specificity and positive and negative predictive values) between sample specimens.

We do not yet have any data on the performance of self-collected anal cytology specimens for a population with a lower prevalence of disease. If a population has a lower prevalence of disease than the population in the present study because it has a higher proportion of HIV-negative men, then this might lead to lower sensitivity of the test for this population. In addition, it is likely that the positive predictive value for such a population would be lower than for our study population and the negative predictive value would be higher.

As with previous studies of anal and cervical cytology, a substantial proportion of normal cytology specimens was falsely negative. In this study, ~30% of subjects with biopsy-proven AIN had normal cytology findings, suggesting that,

similar to cervical screening, sequential anal cytology screening may be necessary to detect AIN in this population. In this context, the role of testing for high-risk human papillomaviruses as an adjunct to anal cytology has not been characterized. Previous studies have shown that detection of multiple human papillomavirus types is a risk factor for progression of low-grade squamous intraepithelial lesions to HSIL, although the risk of progression associated with individual high-risk human papillomavirus types has not been fully addressed.[25]

The findings for self-collected anal cytology specimens were encouraging in this study, although the results should be interpreted with caution. The study participants had experienced the collection of multiple anal cytology samples by clinicians in the past and were aware of how an adequately collected sample felt while being taken. This experience may have enhanced their ability to take an adequate specimen. Self-collected samples were taken 1 month after clinic anal cytology and anal biopsy. It is possible, although unlikely given the slow progression of dysplastic lesions in the cervical and anal canal, that the degree of dysplasia may have changed during this time. It is possible, although unlikely, that taking an anal biopsy specimen could completely have removed the dysplastic lesion or changed the natural history of the lesion. Clinician-collected cytology specimens were obtained using a glass slide and were preserved in ethanol, whereas self-collected specimens were processed using liquid cytology methods. The liquid method may have facilitated improved specimen adequacy by reducing processing problems associated with fecal contamination and low cell count. However, when previously assessed using a split-sample technique, both methods were shown to be comparable, although this may not have been the case in this study.[20]

One of the strengths of this study was that the same pathologist analyzed all of the cytology and biopsy specimens, facilitating comparison of the results. The pathologist was also aware if the cytology specimen was clinician or subject collected because of the different sampling methods used. Therefore, a potential bias may have been introduced into the interpretation of cytology results. However, we do not believe that this affected the results in a substantive way because the pathologist interpreted the samples at least 1 month apart and would not have known the findings for the other samples collected from the same participant.

In summary, these data indicate that self-collected anal cytology specimens, when adequate for interpretation, had sensitivity similar to that of specimens collected by experienced clinicians. The sensitivity of anal cytology sampling to detect AIN 2 and 3 is good among HIV-positive men, but further effort is needed to improve sampling among HIV-negative men. If successful, this technique could greatly facilitate institution of anal cytology screening to prevent anal cancer among at-risk individuals and would be a useful tool to define the prevalence of abnormal anal cytology findings among large populations. Further research is needed to determine whether this technique works as well when performed by individuals with no prior experience with anal cytology and among a population with a lower prevalence of AIN. In addition, the sensitivity, specificity, and positive and negative predictive values should be studied for populations with a lower prevalence of AIN and with a larger number of HIV-negative men.

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APPENDIX 2

Effects of a behavioural intervention to reduce acquisition of HIV infection among men who have sex with men: the EXPLORE randomised controlled study

*The EXPLORE Study Team**

Lancet 2004; 364: 41–50

*Participants and study organisation given at end of paper

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Summary

Background **Effective interventions are needed to prevent acquisition of HIV infection in men who have sex with men. To date, no behavioural interventions specifically for this risk group have been tested with HIV infection as the primary outcome.**

Methods **This multisite two-group randomised controlled phase IIb trial tested the efficacy of a behavioural intervention in preventing HIV infection among 4295 men who have sex with men. The experimental intervention consisted of ten one-on-one counselling sessions followed by maintenance sessions every 3 months. The standard condition was twice-yearly Project RESPECT individual counselling. Twice-yearly follow-up visits included testing for HIV antibody and assessment of behavioural outcomes.**

Findings **The rate of acquisition of HIV infection was 18·2% (95% CI –4·7 to 36·0) lower in the intervention group than the standard group. Adjustment for baseline covariates attenuated the intervention effect to 15·7% (–8·4 to 34·4). The effect was more favourable in the first 12–18 months of follow-up. The occurrence of unprotected receptive anal intercourse with HIV-positive and unknown-status partners was 20·5% (10·9 to 29·0) lower in the intervention than in the standard group.**

Interpretation **The results from the primary analyses allow us to rule out that the experimental intervention is associated with a 35% lower rate of HIV acquisition than in the standard group. The overall estimate of a difference of 18·2%, more favourable estimates of effect in the first 12–18 months, and similar effects on risk behaviours suggest that prevention of HIV infection among men who have sex with men by a behavioural intervention is feasible. Further work should be done to develop more effective interventions.**

Introduction

In 2002, men who have sex with men accounted for 44% of all new diagnoses of HIV infection and AIDS in the USA.[1] The need for interventions to prevent HIV infection with proven efficacy in this risk group is reinforced by the increase in HIV infection among this group during the past 7 years, in contrast to declining rates of risk behaviours and seroincidence observed in the 1980s and early 1990s.[2–10] A recent meta-analysis of behavioural interventions for men who have sex with men showed that interventions focused on interpersonal skills related to risk reduction can reduce self-reported episodes of unprotected anal intercourse.[11] Previous publications also show that interventions should target use of alcohol and recreational drugs, social norms encouraging risk taking, enjoyment of risk-related sexual behaviour, and life events and environments that trigger risk taking.[12,13] These components built into interventions individually tailored to assist a man to move toward risk reduction based on his own and his partners' serostatus, could have the greatest success in reducing risk of infection among men who have sex with men.[14]

The EXPLORE study was designed as a multisite two-group randomised controlled phase IIb or screening trial[15] to test the effect of a behavioural intervention in preventing acquisition of HIV infection among men who have sex with men in the USA. Until this trial, no behavioural interventions specifically for this risk group have been tested in a randomised controlled design with this outcome measure. We aimed to develop and test an intervention that incorporated the best information about effective strategies to address the many factors associated with acquisition of HIV infection among men who have sex with men. Furthermore, we assessed and documented the quality of the intervention delivery to ensure it was delivered according to protocol.

We report the primary study outcomes of the EXPLORE study.

Methods

This study was carried out in six US cities: Boston, MA; Chicago, IL; Denver, CO; New York, NY; San Francisco, CA; and Seattle, WA. Details of the baseline methods and intervention have been published elsewhere.[14,16] Intervention and training manuals, the protocol, outcome measures, and interview details are available from the EXPLORE website (http://www.explorestudy.org). The study was approved by the institutional review boards at each of the participating institutions, and participants provided written informed consent.

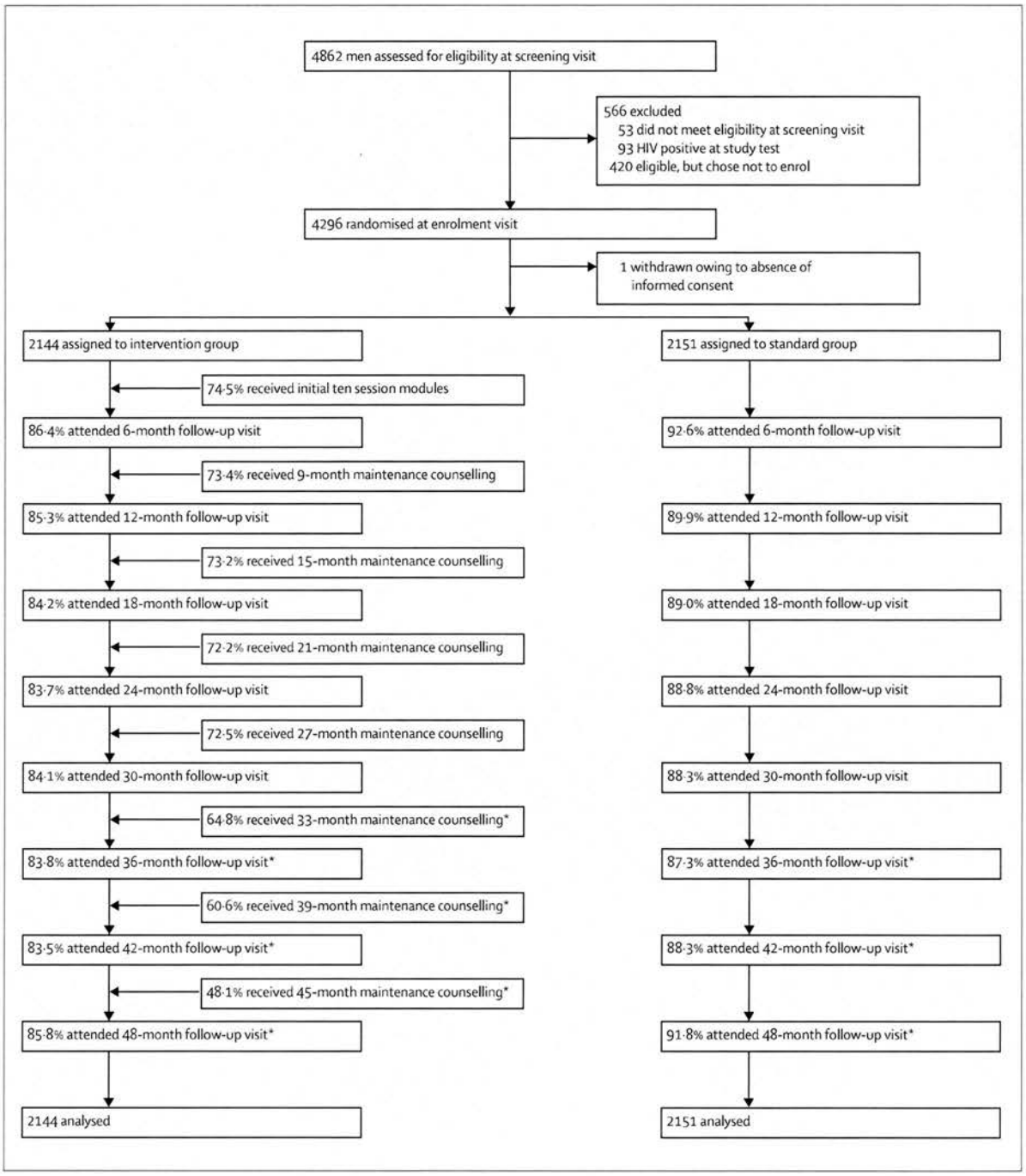


Figure 1: **Trial profile**

*Owing to the common close-out date, percentage is calculated on the basis of participants due at that visit.

Study population

Study participants were recruited at each site between January, 1999, and February, 2001. Recruitment strategies included outreach in streets, at dance clubs, bars, bathhouses, sex clubs, health clubs, and video arcades. Staff within each city also mounted public relations and advertising campaigns, with emphasis on the gay media. Participants also came to the study through internet sites, community forums, community agencies, and referrals from other study participants and clinics. Men were eligible if they were negative for HIV antibodies, were aged 16 years or older, and reported having engaged in anal intercourse with one or more men in the previous year. Men were excluded if they reported a mutually monogamous relationship lasting 2 years or longer with a male partner known to be HIV seronegative.

Design and procedures

Baseline procedures have been described elsewhere.[16] After informed consent had been given, trained interviewers collected information on respondents' demographic characteristics, reasons for participating in the study, history of sexually transmitted diseases, use of postexposure prophylaxis, and history of counselling and psychotherapy. Audio-computer-assisted self-interviewing (ACASI) technology was used to collect information on attitudes towards safer sex, social activities within the gay community, depression, alcohol and drug use, and sexual behaviours. Participants were asked about sexual behaviours in the previous 6 months with partners of each HIV-serostatus type (negative, positive, and unknown). After completing the interviews, participants received HIV pretest counselling, and blood samples were collected for HIV-antibody testing.

About 2 weeks after being screened, participants underwent post-test counselling after receiving the HIV test result. Those with a positive test result at baseline were referred to medical and social services. Men who had no detectable HIV antibodies at the baseline interview were asked to enrol in the trial. The EXPLORE statistical centre generated the allocation sequence to randomise participants to intervention or control conditions in a ratio of one to one (figure 1). Randomisation was stratified by study site and was blocked with random sequences of block sizes. Randomisation was obtained by a telephone call to a voice-prompted, interactive computer program at the statistical centre. Study staff answered questions about the participant's eligibility for randomisation, and the computer verified eligibility, then randomly assigned the participant to one of the two counselling groups.

The experimental intervention, described in detail elsewhere,[14] consisted of ten core counselling modules delivered at one-on-one counselling sessions, typically with one module being delivered per session within 4–6 months of randomisation. For purposes of analysis, the variable "session modules" was defined as the maximum number of modules or sessions completed within 6 months of randomisation. After the initial ten modules, maintenance sessions were delivered every 3 months until the end of the study.

The intervention was designed to address individual, interpersonal, and situation-related factors associated with risk taking among men who have sex with men, such as greater pleasure in or enjoyment of risk-related sexual behaviour, negative mood states, communication difficulties, social norms encouraging misperceptions of risk and risk taking, use of alcohol or recreational drugs, and life events and environments that are catalysts for risk taking. An intervention manual detailed the materials to be covered at each of the ten core behavioural intervention modules. The first three modules established rapport between the counsellor and participant and provided detailed personalised risk assessments to guide the future focus of the intervention sessions. The remaining sessions covered sexual communication, knowledge of personal and others' HIV serostatus in making sexual decisions, and alcohol and drug use in conjunction with risk behaviours. Modules were also offered on how unsafe sex could be triggered by meeting certain types of partners, by places or events related to selection of partners, and by cognitive or emotional cues associated with risk taking. Motivational interviewing was used to help participants make and sustain changes in knowledge, attitudes, beliefs, and behaviours.[17]

The standard condition was twice-yearly counselling on risk reduction based on the Centers for Disease Control and Prevention Project RESPECT model.[18]

Intervention and standard counselling sessions were carried out at the study sites by counsellors who had completed the required 40 h of training. Counsellors were trained to deliver both the standard and the interventional material. Several approaches were used to assess and ensure adherence to the intervention and standard protocols and to keep cross-contamination to a minimum. First, sessions were audio-taped and a planned 10% random sample of tapes (11·5%) were selected for review by raters at the intervention coordinating centre. The sessions were scored on many items specific to the session. The quality-assurance scores were percentages of the total possible score; sessions with scores above 80% were deemed to have followed the protocol and, therefore, to be acceptable. Another approach was to assess the duration of sessions; the intervention sessions were designed to be longer than the standard sessions. Finally, data on quality-assurance scores and duration of sessions were shared with study sites regularly, for monitoring and continuing training purposes, by the clinical coordinator at each site and the intervention coordinating centre.

Follow-up visits were scheduled every 6 months for participants assigned to both study groups. These visits consisted of behavioural surveys with both face-to-face interviews and the ACASI technology. All sexual-behaviour outcomes were collected by ACASI. To mitigate further against participants' under-reporting of risk behaviours owing to social desirability, no study staff had access to any ACASI risk-behaviour information for any participant, including during counselling sessions. Blood samples were collected for testing for HIV antibodies.

Antibodies to HIV were detected by ELISA. Serum samples shown to be reactive after a first test were retested in duplicate. Repeatedly reactive samples were confirmed by western-blot assay or immunofluorescence assay. Participants with a positive test result at any follow-up visit were referred to medical and social services.

Characteristic	Number of participants	
	Intervention (n=2144)	Standard (n=2151)
Age, years		
16–19	43 (2·0%)	50 (2·3%)
20–25	359 (16·7%)	362 (16·8%)
26–30	450 (21·0%)	463 (21·5%)
31–35	458 (21·4%)	452 (21·0%)
36–40	376 (17·5%)	379 (17·6%)
>40	458 (21·4%)	445 (20·7%)
Race/ethnicity		
White	1559 (72·8%)	1553 (72·2%)
Hispanic	322 (15·0%)	330 (15·3%)
African-American	131 (6·1%)	150 (7·0%)
Other	131 (6·1%)	118 (5·5%)
Education		
High school or less	198 (9·2%)	209 (9·7%)
Some college	557 (26·0%)	572 (26·6%)
College	761 (35·5%)	773 (36·0%)
After college	628 (29·3%)	595 (27·7%)
Household annual income, US$		
<12 000	280 (13·1%)	282 (13·1%)
12 000–29 999	579 (27·0%)	587 (27·4%)
30 000–59 999	839 (39·2%)	817 (38·1%)
≥60 000	444 (20·7%)	460 (21·4%)
Currently a student	338 (15·8%)	362 (16·8%)
Employment status		
Full time	1623 (75·7%)	1624 (75·5%)
Part time	208 (9·7%)	218 (10·1%)
Unemployed	219 (10·2%)	208 (9·7%)
Other	94 (4·4%)	101 (4·7%)
Number of male partners in previous 6 months		
0	25 (1·2%)	17 (0·8%)
1	142 (6·6%)	164 (7·6%)
2–5	678 (31·7%)	704 (32·7%)
6–9	393 (18·4%)	357 (16·6%)
≥10	904 (42·2%)	908 (42·2%)
Female sex partner in previous 6 months	86 (4·0%)	92 (4·3%)
HIV-positive male partner in previous 6 months	595 (27·8%)	620 (28·9%)
Anal sex in previous 6 months		
Any unprotected anal sex	1442 (67·7%)	1501 (70·4%)
Unprotected anal sex with positive or unknown-status partner	999 (46·9%)	1049 (49·0%)
Unprotected receptive anal sex with positive or unknown-status partner	598 (28·0%)	608 (28·5%)
Receptive anal sex	1587 (74·2%)	1597 (74·7%)
Unprotected receptive anal sex	1011 (47·4%)	1031 (48·5%)
Insertive anal sex	1731 (80·9%)	1760 (82·1%)
Unprotected insertive anal sex	1135 (53·3%)	1206 (56·5%)
Alcohol and drug use in previous 6 months		
Heavy alcohol use*	234 (11·0%)	219 (10·2%)
Non-injection-drug use	1392 (65·0%)	1382 (64·4%)
Injection-drug use	222 (10·4%)	217 (10·1%)
Depression†	1024 (47·8%)	1006 (46·8%)

Where totals do not reach 2144/2151, data were missing. *Four or more drinks every day or six or more drinks on a typical day when drinking in the last 6 months. †Based on a shortened version of the Center for Epidemiologic Studies depression scale.

Table: **Baseline characteristics by study group**

Statistical analysis

This screening or phase IIb trial was designed to have a high probability of establishing benefit for a highly effective intervention or of ruling out benefit for a totally ineffective intervention. Furthermore, for interventions with lower yet worthwhile efficacy (eg, 35% efficacy), the trial would have high probability of either establishing benefit or indicating plausible efficacy deserving of further study; in that instance, the trial would inform the development of future efficacy trials.

The EXPLORE study was designed so that the intervention strategy would be declared beneficial if the difference in the rate of acquisition of HIV infection between intervention and standard groups was significantly above 10% in favour of the intervention group (that is, that the lower limit of the 95% CI was above 10%). If not, and the difference was significantly below 35% (that is, the upper limit of the 95% CI was below 35%), the benefit of the intervention strategy would be ruled out. In case neither was true, the intervention would be judged plausibly effective with merit for

further evaluation, possibly with refinements. With the target sample size of 4350 and an expected rate of acquisition of HIV infection of 1·55 per 100 person-years in the standard group, if the true difference in the rate of HIV infection was 35% in favour of the intervention group, there would be a 3·0% chance of ruling out benefit, a 50·0% chance of declaring benefit, and a 46·9% chance of stating that the intervention had plausible efficacy. Furthermore, if the true difference in the rate of HIV infection was 0%, there would be a 75·0% chance of ruling out benefit.

By intention to treat, comparisons were made between the participants assigned to the intervention group and those assigned to the standard group, irrespective of the amount of counselling received. The primary analysis was to assess the intervention effect on rate of acquisition of HIV infection. A proportional-hazards model was adopted on the discrete timescale of twice-yearly visits, with the intervention-group indicator as the only covariate. The odds ratio of HIV infection was estimated and the intervention effect was defined as one minus the odds ratio (ie, the percentage of reduction in HIV infection). We assumed in the analysis that HIV serostatus was negative at a missing visit if there were no positive results at earlier visits and an assessment was made at a subsequent visit.

As secondary endpoints, serodiscordant (ie, with HIV-positive or status-unknown partner) unprotected receptive anal intercourse, serodiscordant unprotected anal intercourse, and unprotected anal intercourse were assessed at twice-yearly visits. For each endpoint, its occurrence during the 6 months before a visit was used and a logistic regression model was adopted with visit-independent intervention effect and visit-specific intercepts. The generalised estimating equations approach was used to account for within-participant correlation of repeated outcome measures, with an independent working correlation.

For both primary endpoint and secondary endpoints, additional analyses were done to adjust for baseline characteristics. Specifically, for rate of acquisition of HIV infection, in the proportional-hazards model, we added the following baseline variables as additional covariates: age, injection-drug use, sex with HIV-positive male partner, sex with HIV-positive female partner, unprotected receptive anal sex, and unprotected insertive anal sex, as well as site and race/ethnicity as stratification variables. This step was an attempt to account for imbalances in randomisation and loss to follow-up with respect to baseline characteristics. For serodiscordant unprotected receptive anal intercourse, its baseline measure was added to the logistic regression model as a visit-independent effect. The same approach was used in the adjusted analyses of serodiscordant unprotected anal intercourse and unprotected anal intercourse by adding their respective baseline measures to the regression models.

Before the final analyses, we identified three potential effect modifiers: alcohol use, non-injection-drug use, and depression. Alcohol use was classified as none, light, moderate, or heavy, and non-injection-drug use and depression as yes or no. Subgroup analyses were done for rate of acquisition of HIV infection and occurrence of unprotected anal intercourse, serodiscordant unprotected anal intercourse, and serodiscordant unprotected receptive anal intercourse for each potential effect modifier.

Role of funding sources

The study sponsors participated on the EXPLORE protocol team and provided review of the study design and implementation, including assurance of adherence to good clinical practice in data collection. The sponsors had no role in analysis or interpretation of data or in the writing of the report.

Results

Of the 4862 men screened for the study, 4296 enrolled and were randomly assigned to the two study groups (figure 1). The final analysis was based on 4295 participants; one participant was randomised in the absence of informed consent.

The mean age of the participants was 34·0 years (SD 9·4) and 19·0% were 25 years of age or younger. 72·5% of the participants were white, 15·2% were hispanic, and 6·5% were African-American. 35·8% had education less than a college degree, and 40·3%

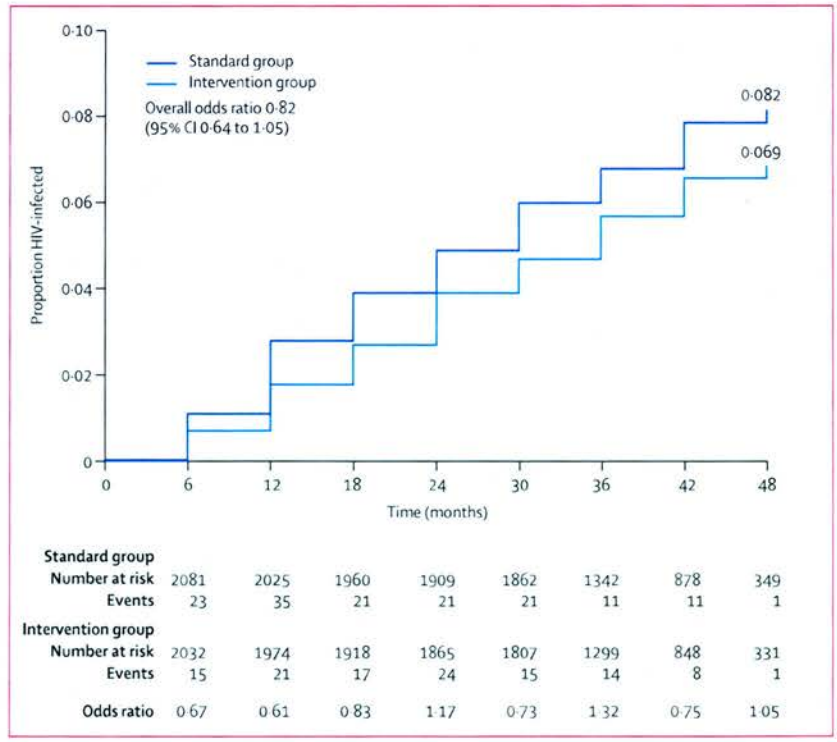


Figure 2: Kaplan-Meier curve of proportion of participants infected with HIV

had an annual household income of less than US$30 000. The enrolled men reported a median of seven male sex partners in the 6 months before screening (IQR three to 18); however, 42·2% reported having ten or more male partners and 28·4% having an HIV-infected partner. 69·1% reported any unprotected anal intercourse, 48·0% reported unprotected receptive anal intercourse, and 54·9% reported unprotected insertive anal intercourse. 47·9% reported unprotected anal intercourse with an HIV-positive or unknown-status partner, and 28·2% reported unprotected receptive anal intercourse with an HIV-positive or unknown-status partner. Heavy alcohol use was reported by 11·0% of participants and any non-injection-drug use by 64·7%. At baseline, 47·3% of men had symptoms of depression as found by a shortened version of the Center for Epidemiologic Studies depression scale. Baseline characteristics were well balanced between the intervention and standard groups, with the exception of unprotected insertive anal intercourse (table).

Of the 2144 men assigned the intervention, 1598 (74·5%) completed all ten initial session-modules, 154 (7·2%) completed seven to nine, 105 (4·9%) completed four to six, 258 (12·0%) completed one to three, and 29 (1·4%) did not complete any. More than 70% of the men in the intervention group received maintenance sessions except at the 33-month, 39-month, and 45-month visits (figure 1).

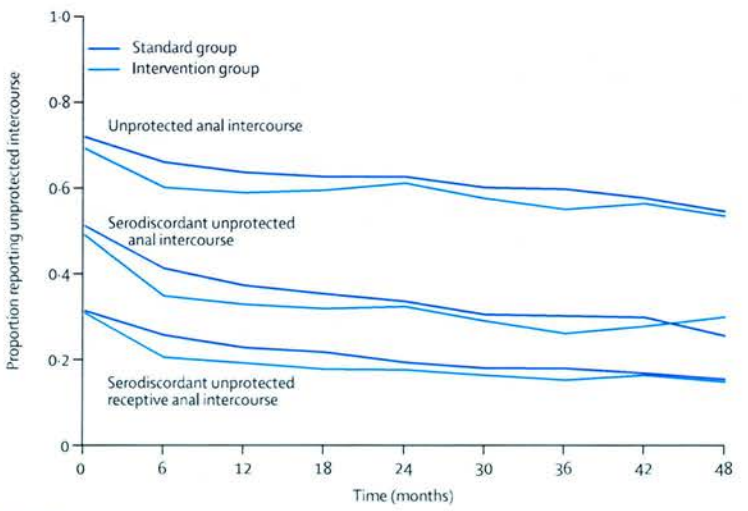
Of the initial ten sessions, 1691 were reviewed and 83·3% met the quality-assurance criterion of a score of 80% or higher. 651 maintenance visits for the intervention group were also reviewed and 77·1% met this criterion. The proportion of the 6-monthly visits that met the quality-assurance criterion did not differ significantly between the intervention and standard groups (76·9% of 657 reviewed *vs* 80·7% of 841 reviewed, p=0·0677). The mean duration of the sessions at the 6-monthly visits was 16·1 min longer for intervention sessions than for standard sessions (37·9 [SD 20·4] *vs* 21·8 [11·1] min; p<0·0001).

Visit retention rates were above 83% in the intervention group and above 87% in the standard group during the follow-up period (figure 1). Retention was consistently higher in the standard group than in the intervention group. Lower retention, as defined by final-visit retention status, was significantly associated with minority-group status (89·5% white men retained *vs* 83·9% others), younger age (89·8% >25 years *vs* 80·0% <25 years), reporting of female sex partners at baseline (88·5% no female partners *vs* 74·2% one or more female partners), and reporting of unprotected receptive anal intercourse at baseline (89·0% no unprotected receptive anal intercourse *vs* 86·7% reporting unprotected receptive anal intercourse). In the intervention group, retention also was significantly associated with completion of the initial ten session-modules (92·2% for nine or ten session-modules *vs* 63·6% for less than nine session-modules).

The overall rate of acquisition of HIV infection in the study cohort was 2·1 per 100 person-years (95% CI 1·9 to 2·4). There were 6037 person-years of follow-up in the intervention group and 6203 in the standard group. 115 intervention-group participants and 144 standard-group participants became infected with HIV during the study. By intention-to-treat analysis, the odds ratio was 0·82 (95% CI 0·64 to 1·05) for the intervention group relative to the standard group (figure 2). Thus, the difference in the rate of acquisition of HIV infection was 18·2% (95% CI –4·7 to 36·0) in favour of the intervention group. The data suggest that the difference in acquisition of HIV infection was greatest in the first 12–18 months of the study (figure 2).

After adjustment for study site and baseline characteristics associated with retention and distributed differently in the two study groups, the estimated odds ratio for the intervention was 0·84 (0·66 to 1·08), translating to a 15·7% (–8·4 to 34·4) difference in HIV acquisition in favour of the intervention group. Subgroup analyses by baseline alcohol use, non-injection-drug use, and depression score were consistent with the overall results (data not shown).

The estimated odds ratios of reporting unprotected anal intercourse and serodiscordant unprotected anal intercourse in the intervention group relative to the standard group (figure 3) were 0·86 (0·79 to 0·94) and 0·85 (0·78 to 0·94). These odds ratios translate to differences in favour of the intervention group of 13·9%



Odds ratio									
SDURA	0·98	0·71	0·77	0·74	0·88	0·87	0·78	0·96	0·94
SDUA	0·92	0·75	0·81	0·85	0·94	0·92	0·80	0·89	1·28
UA	0·88	0·77	0·81	0·87	0·94	0·90	0·82	0·95	0·96

ure 3: **Proportions reporting unprotected intercourse by study group**
URA=serodiscordant unprotected receptive anal intercourse; SDUA=serodiscordant unprotected anal ercourse; UA=unprotected anal intercourse.

(5·6 to 21·5) in unprotected anal intercourse and 14·8% (6·3 to 22·5) in serodiscordant unprotected anal intercourse. We also examined the highest risk behaviour, unprotected receptive anal sex with a partner positive for HIV or of unknown status; the estimated odds ratio was 0·80 (0·71 to 0·89), translating to a difference of 20·5% (10·9 to 29·0).

After adjustment for study site and baseline characteristics associated with retention and distributed differently in the two groups, the efficacy estimates for unprotected anal intercourse, serodiscordant unprotected anal intercourse, and serodiscordant unprotected receptive anal intercourse were 13·2% (4·8 to 20·9), 14·8% (6·5 to 22·4), and 22·5% (13·3 to 30·7). Subgroup analysis of the sexual behavioural outcomes showed similar results to overall results (data not shown).

Discussion

Challenges for trials of behavioural interventions have been completion of the intervention and retention of study participants over time.[19] Most participants in the EXPLORE intervention group received all ten initial session-modules and maintenance sessions, and the sessions were delivered with high conformity with the protocol. Visit retention was above 87% in the standard group and above 83% in the intervention group over the 4-year study.

The primary analysis found that the intervention was associated with an 18·2% lower rate of acquisition of HIV infection; an analysis adjusted for baseline covariates attenuated the intervention effect to 15·7%. Analysis of the behavioural outcomes found that the intervention significantly affected the occurrence of the highest risk behaviour,[20] unprotected receptive anal intercourse with HIV-positive and unknown-status partners, as well as unprotected anal intercourse with HIV-positive and unknown-status partners, and unprotected anal intercourse.

The results from the primary analyses allow us to rule out that the experimental intervention lowers the rate of HIV acquisition relative to the standard group by 35%. The overall estimate of an 18·2% difference between the groups, together with more favourable estimates of effect in the first 12–18 months and similar changes in risk behaviours, suggests that a behavioural intervention can prevent HIV infection, not just reduce the frequency of self-reported risk behaviours. Further analyses of our data are under way with the aim of generating hypotheses to help develop more effective behavioural interventions. For example, are there subpopulations of men who have sex with men with demographic characteristics, baseline risk behaviours, or psychosocial measures for whom the behavioural intervention was more beneficial? With such evidence, more targeted behavioural interventions could be developed and tested. Furthermore, although over 70% of men completed the ten-session intervention in this study, other designs of such interventions should be explored to improve the acceptability to different groups and facilitate implementation in community settings.

We are aware of only one other trial of an intervention to reduce HIV risk for men who have sex with men that involved a biological endpoint.[21] In that study, the intervention was a 1-day workshop combining several models of behaviour change with motivational interviewing strategy. The primary infection outcome was a new sexually transmitted disease, as assessed from clinical and laboratory databases, and behavioural outcomes were collected by mailed questionnaire. Contrary to expectations, over 12 months of follow-up, a larger proportion of men in the intervention group than in the control group receiving standard care had a new sexually transmitted disease. Behavioural outcomes did not significantly differ between the groups.[21]

Two other large-scale trials of behavioural interventions with biological outcomes have been carried out in the USA among predominantly heterosexual populations. The Project RESPECT trial tested individually delivered interactive risk-reduction interventions delivered in either two or four sessions and found, over 12 months, a 20% lower rate of new sexually transmitted diseases than with people receiving didactic messages. The effect of the intervention was strongest early in follow-up.[18] The National Institute of Mental Health Multisite HIV Prevention Trial of a seven-session small group intervention found no difference between study groups in overall rate of reinfection with sexually transmitted diseases over 12 months.[22] Other smaller randomised trials of behavioural interventions have been done with sexually transmitted disease outcomes among women, patients with sexually transmitted diseases, and adolescents, with only one demonstrating a significant effect on rates of sexually transmitted disease.[19,23] The lower rate of HIV acquisition with the intervention in the first 18 months of follow-up in EXPLORE was similar to that found with sexually transmitted diseases in Project RESPECT. Furthermore, the EXPLORE study found similar effects on risk behaviours over 12–18 months of follow-up to Project RESPECT and the meta-analysis of HIV intervention studies among men who have sex with men.[11]

The EXPLORE trial had several limitations. First, the study sample recruited is not necessarily representative of men who have sex with men in the participating cities. Eligibility criteria were established to enrol a high-risk HIV-antibody-negative population for a trial with an HIV-infection endpoint. Furthermore, generalisability is limited since black and hispanic men, younger men, and those of lower socioeconomic status were less likely to enrol in the study, were more likely not to be eligible for the study owing to behavioural characteristics, and were more likely to be HIV infected[16] and not retained in follow-up than white men in the study. However, the

population recruited was clearly at high risk as shown by the rate of acquisition of HIV infection and self-reported behaviours, which emphasises the need for prevention efforts to continue for many subpopulations of men who have sex with men. Second, the full effect of the intervention may have been muted for several reasons. The standard condition was based on the best available model, the Project RESPECT two-session behavioural intervention model, and decreases in sexual risk behaviours were observed in both study groups in the EXPLORE study. The amount of counselling in the standard group was probably more than that given in most public health settings and not equivalent to usual care, in which individuals would voluntarily seek anonymous counselling and testing in many cases, or to typical HIV counselling delivered in the community. In addition to the counselling, participants in both groups received much attention during the study, including repeat HIV-antibody testing, scheduling, and reminder letters or calls for all visits, newsletters, and other activities to maintain involvement and retention. Another possible reason for a muted intervention effect is cross-contamination if participants in the control group were in contact with others who were part of the intervention group. This effect, however, was likely to be negligible because only 13% of the men were enrolled as referrals from friends. Other community HIV-prevention efforts available to participants in both groups could also affect the results. Finally, the full effect of the intervention could have been dampened because not all intervention-group participants received the full initial ten session-modules or attended all maintenance visits. However, only 13% of the intervention-group participants received three or fewer of the initial session-modules.

A third limitation was a differential in retention between the study groups, with 90% and 86% retained at their final visit in the standard and intervention groups, respectively; those not retained tended to be from higher-risk subgroups. This higher rate of non-retention in the intervention group can be explained by the low retention rate in the participants who had lower adherence to the initial ten session-modules. Although the men in the intervention group who completed at least nine of the initial ten session-modules had a retention rate of 92%, close to that achieved in the control group, the remainder of the intervention group had only 64% retention. These data suggest that approaches are needed to improve the capture of longer-term outcome results, particularly among participants who have lower adherence to behavioural interventions.

Other research has shown that self-reported behavioural outcomes can overestimate the benefit of behavioural interventions and that the relation between the frequency of reported sexual behaviours and rate of HIV infection is probably complex and dependent on factors related to selection of partners and background prevalence of HIV infection.[19,21] The use of ACASI technology in our study might have helped to limit potential problems with the validity of self-reported behaviours.[24,25]

The challenge for behavioural interventions has been maintenance of behaviour change over extended periods. The EXPLORE study was done during a period of substantial changes in the prevalence of risk behaviours and HIV incidence in communities of men who have sex with men, probably related to widespread use of highly active antiretroviral treatments and shifts in social norms.[9,26,27] This trial provides encouragement that behavioural interventions can achieve reductions in risk in the short term. However, achievement of long-term effects is one of the most challenging features to face in design of effective behavioural interventions. In the long run, engagement of individuals at high risk in intensive interventions over a short period and delivery of periodic "boosters" may not be effective. A different model should be considered for long-term behavioural change, possibly combining improved, individualised interventions with community and structural changes to encourage and support behavioural change, particularly in relation to unprotected anal sex and disclosure of HIV serostatus.

Contributors

B Koblin, M Chesney, and T Coates conceived the study, oversaw all features of its implementation, and formed the writing committee. M Husnik, G Beauchamp, and Y Huang provided statistical expertise and did the data analyses. S Bozeman and M Madison were responsible for overall protocol implementation. S Buchbinder, C Celum, G Colfax, F Judson, B Koblin, K Mayer, and D McKirnan supervised study implementation at the individual research sites. M Chesney and T Coates supervised study activities at the study intervention coordinating centre. All other staff contributed to protocol implementation.

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Conflict of interest statement

None declared.

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APPENDIX 3

Section 8 Protocol

HPV PROTOCOL VERSION 3.0 SUMMARY

Title:	Anal HPV Infection and Neoplasia as Risk Factors for HIV Seroconversion
Participants:	MSM recruited into HIVNET 015 (Explore) at risk of HIV infection.
Design:	A multi center, prospective study of HPV infection, cytological abnormality and HIV seroconversion.
Estimated Time:	Enrollment period ends July 31, 2002. For each participant enrolled at 12, 18, and 24 Month study visits, there is an 18-month follow-up (one baseline and three semi-annual visits) and for participants enrolled at 30 Month study visit, a 12-month follow-up (one baseline and two semi-annual visits).
Period:	1/31/01-7/31/03
Sponsoring Agency:	State of California University-Wide AIDS Research Program
Study Coordination and Monitoring: (PPDI)	Abt Associates Inc.- Protocol Coordination Team (PCT) Pharmaceutical Product Development, Inc.- Study Monitor
Study Sites:	Boston, Denver, New York, and San Francisco
Data Analysis:	Eric Vittinghoff, Ph.D. San Francisco Department of Public Health
Data Coordination:	Statistical Center for HIV/AIDS Research and Prevention (SCHARP)
Central Laboratory:	Laboratory of Dr. Joel Palefsky Department of Laboratory Medicine University of California San Francisco San Francisco, California
Repository:	N/A
Laboratory	N/A

1.0 INTRODUCTION

1.1 Background

Human papillomavirus (HPV) infection is one of the most common sexually transmitted agents among both men and women, and HPV-associated anal condyloma is one of the most common sexually transmitted diseases among MSM practicing receptive anal intercourse. HPV has received much attention in the last decade due to its causal association with anogenital squamous cell cancer, including cancer of the cervix, vulva, vagina, penis and anus.

There are two important reasons to study anal HPV infection and HPV-related lesions in the anal canal of MSM. The first is that anal HPV infection may lead to development of anal cancer and its putative precursors, anal squamous intraepithelial lesions (ASIL). An understanding of the biology of HPV infection and ASIL is critical to the development of an effective strategy for prevention of anal cancer in MSM, which as described below, is relatively common and potentially preventable. The second reason is perhaps even more important, which is that anal HPV infection and ASIL may represent important risk factors for the acquisition of HIV infection among HIV- men, as well as new strains of HIV among men who are already HIV+.

A) Anal HPV infection and ASIL as risk factors for anal cancer

The primary reason to expand our knowledge about anal HPV infection and ASIL is their putative relationship with invasive anal cancer. Anal cancer and its precursors among MSM have been the focus of study for a number of years. The reason for this focus is that the incidence of anal cancer among MSM was approximately 35/100,000 prior to the HIV epidemic (1, 2). Therefore the incidence of anal cancer in this group prior to the HIV epidemic was similar to that of cervical cancer in women prior to the introduction of cervical cytology screening and several-fold higher than current rates of cervical cancer in women (3). Historically, most anal cancers in men and women occur after the age of 50 but anal cancer has recently been increasing among younger men. The relationship between anal cancer incidence and the HIV epidemic is not yet clear. One study suggested that the increase in anal cancer among single, never-married men in San Francisco between the ages of 25 and 44 years predated the HIV epidemic (4). In contrast, others have shown that the rate of invasive anal cancer increased with increasing proximity to an AIDS diagnosis, suggesting an association between anal cancer and HIV-related immunosuppression (5).

The term "ASIL" encompasses a wide range of HPV-related changes in epithelial cells. At one end of the ASIL spectrum are "condyloma" and "mild dysplasia" or "anal intraepithelial neoplasia 1" (AIN 1). Analogous to the Bethesda system of grading cervical cytology (6), we, as well as others, have consolidated these into low-grade squamous intraepithelial lesions (LSIL). At the other end of the ASIL spectrum, the terms "AIN 2" (moderate dysplasia) and "AIN 3" (severe dysplasia) have been consolidated into high-grade squamous intraepithelial lesions (HSIL). Each of these categories is diagnosed using the same criteria as those used for cervical disease (7). HSIL is considered to be the true cancer precursor lesion and is always treated to prevent cancer when possible. While LSIL is not considered pre-cancerous *per se*, and is usually not treated, it may progress to HSIL. Another grade of cytologic abnormality known as "atypical squamous cells of undetermined significance" (ASCUS) may also be found. ASCUS is between normal and LSIL in its

association with HPV infection and concurrent presence of ASIL on anoscopy and we customarily include ASCUS in the "abnormal" cytology category.

The paradigm for the clinical significance of ASIL and its relationship to invasive anal cancer is cervical SIL (CSIL) and invasive cervical cancer. The basis for this parallel is four-fold: 1) the presence of a transformation zone in the anal canal similar to that of the cervix. Many HPV-related lesions of the cervix arise in the cervical transformation zone, the site where the columnar epithelium of the endocervix meets the squamous epithelium of the exocervix. A similar area is present in the anal canal at the anorectal junction, where the columnar epithelium of the rectum meets the squamous epithelium of the anal canal; 2) ASIL is histologically quite similar to CSIL. Invasive anal cancer likewise is histologically similar to invasive cervical cancer. As with invasive cervical cancer, many cases of invasive anal cancer have overlying HSIL suggesting that HSIL is the lesion from which the cancer arose; 3) the relationship between HPV and anal disease has been documented in a number of publications (7-9) and is similar to the relationship between HPV and cervical disease. As in the cervix, HPV 16 is the most common HPV type found in association with anal cancer and there are few anal cancer cases with only the "benign" HPV types such as 6 or 11 (7, 8) and 4) the range of HPV types found in the anal canal is similar to that of the cervix (10). Since development of cervical cancer is preventable through detection and treatment of CSIL before it progresses to cancer, it is highly likely that treatment of ASIL will prevent development of anal cancer. Anal cancer is thus one of the few preventable malignancies in either HIV+ or HIV- men, and an understanding of the natural history of ASIL and associated risk factors is clearly needed to develop appropriate prevention strategies.

For the last five years, we have performed studies of the prevalence of anal HPV infection and ASIL in a cohort of 346 HIV+ and 242 HIV- men, as well as changes in infection and disease over a 4-year follow-up period. The results of these studies are summarized in the Preliminary Data section and in manuscripts in the appendices. Because the subjects were enrolled primarily from the San Francisco Men's Health Study, the mean age at enrollment of the HIV+ men was 42 years (range, 24-64) and the mean age of the HIV- men was 45 years (range, 26-73). A high proportion of HIV+ and HIV- men had anal HPV infection and ASIL at baseline and after 4 years of follow-up; the projected incidence of HSIL was 49% among HIV+ men and 17% among HIV- men. These data indicate that a large proportion of both HIV+ and HIV- men are at risk of developing anal cancer.

This study will be performed in HIV- men, but will also have important implications for HIV+ men. Since a high proportion of HIV- men had anal HPV infection at baseline, we believe that many HIV+ men will have acquired HPV infection and possibly ASIL prior to acquisition of HIV infection. An understanding of anal HPV infection and ASIL among HIV+ men is therefore dependent on understanding the dynamics of infection and disease prior to seroconversion.

Risk factors for acquisition of HPV infection and development of ASIL also remain poorly understood. In our cohort study described above, we did not have a sufficient number of HIV- men to perform multivariate analyses of risk factors. Even more importantly, since most of the men were in their forties, we believe that they may have acquired HPV infection too long ago to provide meaningful data. For this

reason, studies of HIV- men younger than age 26 provides an ideal opportunity to better understand risk factors for HPV infection and ASIL. To illustrate this point, we have been studying anal HPV infection in high-risk HIV+ and HIV- women, mostly age 35 or older. When queried about receptive anal intercourse as a risk factor for HPV infection and ASIL, there was no clear association. However, when we repeated this study among adolescent women who had recently initiated sexual activity, we found anal HPV infection in nearly 30%, and there was a clear correlation with receptive anal intercourse in this group.

B) Anal HPV infection and ASIL as risk factors for HIV seroconversion
Several studies have documented the role of sexually transmitted diseases (STDs) as cofactors for HIV acquisition (11-14). To date there have not been any rigorous studies of the role of HPV infection or ASIL in potentiating HIV acquisition. The role that STDs play in HIV acquisition may be twofold. First, these STDs are often associated with mucosal friability and the increased possibility of body fluid exchange, including blood, may enhance HIV transmission. Second, these STDs lead to inflammatory responses, and the increased presence of lymphocytes prone to HIV infection may also potentiate HIV acquisition. HPV-related diseases may lead to HIV acquisition for both of these reasons. In our experience, men who have internal anal lesions, whether low-grade or high-grade, bleed very easily when a swab is passed into the anal canal to obtain specimens for HPV testing or cytology. Insertion of objects into the anal canal, as in receptive anal intercourse, may similarly lead to more frequent and larger volumes of bleeding if ASIL is present. Biopsies of ASIL frequently show a lymphocytic infiltration, similar to cervical SIL lesions in which the majority of the lymphocytes in cervical lesions are CD4 cells. Therefore it is likely that cells susceptible to HIV infection are present in larger quantities in ASIL lesions, at sites that are also likelier to bleed. For both these reasons, we believe that HPV infection and ASIL increase the risk of HIV acquisition among HIV- men.

Additionally, HIV+ men who have ASIL at the time of engaging in receptive anal intercourse may be at risk of exposure to new HIV strains and may also be likelier to transmit HIV to active partners. Since women have been shown to acquire cervical HPV infection soon after initiation of sexual activity, we hypothesize that MSM also acquire HPV infection soon after initiating receptive anal intercourse. If so, then they may have HPV infection and possibly ASIL at a time of their lives when they may be most likely to practice unsafe sex with a large number of partners. If our data do show that ASIL is a risk factor for HIV acquisition, this may have important implications for development of new strategies to prevent HIV transmission. Specifically, detection and treatment of ASIL in high-risk HIV- MSM may be useful to lower the risk of HIV acquisition.

In summary, more information is needed on the biology of anal HPV infection and ASIL among MSM. These data are needed to better understand HPV infection and ASIL as risk factors for anal cancer in both HIV- and HIV+ men. They are also needed to determine if HPV infection and ASIL are risk factors for HIV acquisition.

1.2 Rationale
Anal cancer is a relatively common malignancy among men who have sex with men (MSM) and may be even more common among HIV+ MSM. In the past few years, we have collected much data on anal HPV infection and anal squamous intraepithelial

lesions (ASIL) from a cohort of 346 HIV+ and 262 HIV- MSM. These data show that anal HPV infection and ASIL are very common among men in the 35-45 year age range. Our follow up of the cohort showed that the 4-year projected incidence of the putative anal cancer precursor lesion high-grade squamous intraepithelial lesion (HSIL) was 49% among HIV+ men and 17% among HIV- men. While men with anal HPV infection and ASIL are probably at increased risk of anal cancer, little is known about the prevalence or incidence of HPV infection and ASIL in men at either end of the age spectrum, i.e., men over the age of 50 years and men aged 25 years or less. Although these studies will be performed in HIV- men, but will also have important implications for HIV+ men, since we believe that many HIV+ men will have acquired HPV infection and possibly ASIL prior to acquisition of HIV infection. An understanding of anal HPV infection and ASIL among HIV+ men is therefore dependent on understanding the dynamics of infection and disease prior to seroconversion. Knowledge of the natural history of anal HPV infection and ASIL in the early years after sexual activity begins will therefore be very important. Like cervical cancer, anal cancer may be preventable through screening for and treatment of ASIL, and data collected in this study could have important implications for development of anal cancer screening programs for both HIV+ and HIV- men.

1.3 Study design overview

Our proposed study will characterize HPV infection and anal cytologic abnormalities in 1400 to 2000 MSM chosen from among 2900 men recruited into four of six HIVNET 015 (Explore) study sites. Enrollment into HPV ancillary study ends July 31, 2002.

At 12, 18, 24 and 30 months after enrollment to HIVNET 015 (Explore), and at 6-month intervals for the duration of this study, we will obtain a minimum one anal swab from each participant. The swab will be for anal cytology [per our standard protocol] using the Thinprep method as well as for HPV testing.

In designing the behavioral intervention study, the investigators assumed that the HIV seroincidence during the planned follow-up period would be approximately equal to that observed in VPS (1.55/100-person years). These seroconversion rates were used in determining sample size calculations for our proposed study examining the association between HPV infection, ASIL and HIV seroconversion. Since one half of our study subjects will be in the intervention group, for the purposes of sample size determination, we have assumed that the intervention will be successful in reducing HIV seroconversion by 30%.

2. STUDY OBJECTIVES

The following objectives will be addressed:

2.1 To study the prevalence, incidence and risk factors for anal human papillomavirus infection in a cohort of sexually active HIV- MSM.

2.2 To study the prevalence, incidence and risk factors for anal cytologic abnormalities in a cohort of sexually active HIV- MSM.

2.3 To study the association between HIV seroconversion and detection of anal cytologic abnormalities or anal HPV infection in a cohort of sexually active HIV-MSM.

3. STUDY DESIGN

Our proposed study will characterize HPV infection and anal cytologic abnormalities in 1400 - 2000 MSM chosen from among 2900 (four out of six sites) men recruited into HIVNET 015. All eligible participants will be offered enrollment over a maximum of a two-year accrual period until a total of 1400 - 2000 men are enrolled. Study enrollment ends July 31, 2002.

HIVNET 015 participants will be offered enrollment in the HPV study at the Month 12 study visit, unless the participant has been on study more than 486 days (closing date of the Month 12-visit window) when the study is implemented. Thus some participants will be offered enrollment at the Month 18 study visit, and a few will be offered enrollment at the Month 24 study visit and Month 30 study visit.

At HPV study enrollment and at three 6-month interval visits (or two 6-month interval visits for Month 30 enrollees) and for the duration of this HPV study, we will obtain a minimum of one anal swab from each participant. The swab will be used for anal cytology [per our standard protocol] using the Thinprep method as well as for HPV testing.

4. PARTICIPANT ELIGIBILITY

This study will include up to 2000 men at high risk for HIV infection enrolled in the HIVNET 015: A Randomized Clinical Trial of the Efficacy of a Behavioral Intervention to Prevent Acquisition of HIV Among Men who have Sex with Men.

4.1 Inclusion criteria

Persons may be included in the study if they meet the following criteria

- Enrolled in the HIVNET 015 (Explore)
- Willing and able to provide written informed consent.

4.2 Exclusion Criteria

Persons will be excluded from the study if they:

- Have an obvious psychological/psychiatric disorder that would invalidate the informed consent process, or otherwise contraindicate participation in the study.
- Having any other condition that in the opinion of the study site Principal Investigator will interfere with achieving the study objectives.

4.3 Conditions for withdrawal

Participants may withdraw from study participation at any time, for any reason, without loss of other benefits or services to which they are entitled. Withdrawal from

the HPV ancillary study does not affect participation in the HIVNET 015 (Explore) trial.

Participants may be withdrawn from the study due to inability to comply with study procedures. Participants also may be withdrawn at the request of the study site investigator, following review with the co-chairs, protocol bio-statistician, and the Protocol Coordination Team (PCT) Project Officer.

5. STUDY PROCEDURES

An overview of the study visit and procedure schedule is presented in Table 1.

Table 1. Schedule for visits and procedures during the study.

D = HPV study visits for all enrolled participants
D_1= HPV study visits for those who enroll at the Month 12 HIVNET 015 visit only
D_2= HPV study visits for those who enroll at the Month 18 HIVNET 015 visit only
D_3= HPV study visits for those who enroll at the Month 24 HIVNET 015 visit only
D_4= HPV study visits for those who enroll at the Month 30 HIVNET 015 visit only

Study Month – HIVNET 015	**12**	**15**	**18**	**21**	**24**	**27**	**30**	**33**	**36**	**39**	**42**
Enrollment in HPV Study	D_1		D_2		D_3		D_4				
Laboratory tests											
Anal Cytology	D_1		D		D		D		D_2 D_3 D_4		D_3 D_4
Anal HPV Testing	D_1		D		D		D		D_2 D_3 D_4		D_3 D_4
Counseling											
Anal Cytology and HPV Testing	D_1		D		D		D		D_2 D_3 D_4		D_3 D_4

Remote Participation: Remote participation is not offered in the HPV ancillary study. If a participant moves to a non-HPV HIVNET 015 (Explore) study site city, we will not ask them to continue in the HPV study. An HPV ancillary study termination form will filled out for participants who move and are unable to complete visits at an HIVNET 015 (Explore) HPV ancillary study site.

5.1 Consent

The template for the consent form for the HPV ancillary study is in the HPV SSP Section 10, Appendix. Each participant must sign the consent form prior to participating in the HPV study.

5.2 Specimen collection

Once the study is discussed with the subject and the consent form is signed.

1) Moisten Dacron™ swab (Dacron swab to be provided by J. Palefsky) in tap water and insert swab into the anus as far as it will go
2) Remove swab slowly with gentle pressure on the walls, rotating the Q-tip as you withdraw.
3) Insert swab into the Cytyc bottle, vigorously swirling the swab in the buffer and squeezing the swab against the sides of the bottle.
4) Discard the swab.
5) Cap the bottle very tightly to prevent leakage. Label the bottle with the labels provided and the date the specimen was collected.
6) Store Cytyc cytology bottles at room temperature until shipment. Specimens may be stored for up to one week and shipped Monday to Thursday.
7) Ship all specimens in the provided containers to:
   Maria DaCosta
   521 Parnassus Ave
   Room C233
   University of California San Francisco
   San Francisco, CA 94143

5.3 Follow-up of anal cytology results

The study protocol calls for anal cytology results to usually be made available to subjects within 2 months (and no longer than 4 months after collection) of their visit. If the cytology shows HSIL or cancer (we expect this to happen very rarely if at all), then the subject must be notified as soon as those results are available. Questions about the results may be addressed to Dr. Joel Palefsky or Dr. Peter Chin-Hong through a toll free 800 number. That number is 1-888-345-1588.

Other contact information for Dr. Palefsky is:
Dr. Joel Palefsky
Tel: 415-476-1574
Fax: 415-476-4204
E-mail: joelp@medicine.ucsf.edu

6. LABORATORY SPECIMENS

Specimens will be collected for testing at the Department of Pathology, UCSF (cytology samples) and the Department of Laboratory Medicine, UCSF (HPV samples).

6.1 Anal Cytology Testing

In this study, Thinprep anal cytologic smears will be prepared according to the manufacturer's instructions. Our pathologist, Dr. Teresa Darragh, who is an expert in gynecologic pathology and is one the most experienced pathologists in the world in interpreting anal cytology and histopathology, will read each slide. She will interpret the slides independently and without knowledge of the results of anal HPV DNA testing. The criteria for grading anal cytologic smears will be identical to those for cervical cytology (15).

6.2 HPV testing protocols

The approach that we use for HPV testing uses PCR because it is an extremely sensitive test that provides information on the presence of 39 different HPV types. This test detects both high and low level infections. By determining the presence of each type individually, we can also analyze other combinations such as HPV 16 and 18 together. We will determine how often these individual types persist and whether we can detect new types over time.

To perform PCR, an aliquot of the Cytyc specimen is removed from each specimen when the bottle is opened for the first time. The specimens are heated at 56°C for one hour to inactivate HIV and digested with 10 mg/ml proteinase K. The PCR protocol that we use was adapted from a method by Ting et al and is being used by others and us for many different studies (16). The sample for PCR is added to a tube containing one ml ammonium acetate/EtOH solution, and the DNA is allowed to precipitate at -20°C overnight or at -70°C for one hour. Thirty five cycles of amplification are performed. Detection of the PCR products is performed using a non-radioactive enhanced chemiluminescence (ECL) dot blot technique. Each membrane contains a set of negative L1 hybridization controls that include human HPV-negative cells and specimens containing all the components of the reaction except target DNA. Positive controls consist of plasmid amplifications of the different HPV types. Each membrane also contains five specimens chosen at random to be amplified and probed in duplicate, and therefore 5% of our specimens are tested twice as a check for assay reliability. The PCR product is fixed to the membrane in a UV-crosslinker. ECL detection is performed according to the manufacturer's (Amersham) instructions, and the blots are exposed to X-ray film at room temperature for 15 minutes and two hours. The blots are probed with a generic probe mix consisting of biotinylated HPV L1 PCR products from HPV 11, 16, 18, and 51. A second membrane is probed with ß-globin oligonucleotide probe to detect the presence of human DNA and the integrity of the amplification reaction. Ten more membranes containing specimens positive with the generic probe are made and probed with each of the following types individually: 6, 11, 16, 18, 26, 31, 32, 33, 35, 39, 40, 45, 51, 52, 53, 54, 55, 56, 58, 59, 61, 66, 68, 69, 70, 73, AE2, Pap 155, Pap 291, and the following types together in a probe mixture: HPV 2, 13, 34, 42, 57, 62, 64, 67, 72, W13B. The presence of a dot on the membrane with a probe for a given HPV type indicates positivity for that type. If a specimen is negative for ß-globin, this implies that the specimen contains an inadequate number of cells or an inhibitor of the amplification reaction. HPV results from these specimens are excluded from analysis.

Specimens that are positive with the generic probe, but negative for the 39 specific types are considered to have an "unknown" HPV type. Because of its high sensitivity, PCR by definition must be performed with great care to avoid the possibility of contamination. We take all possible precautions in handling our samples, including separation of pre- and post-amplification products in different parts of our research building. If any of the above negative and positive controls do not work, the reaction is repeated. We perform a large volume of HPV testing for this study and other studies and to date we have not have problems with contamination.

7. EVALUATION OF OUTCOMES

The outcomes to be measured are incidence of anal HPV infection, incidence of anal cytological abnormality, prevalence of anal HPV infection, prevalence of anal cytological abnormality, incidence of HIV infection, demographics of anal HPV infection/HIV infection/anal cytological abnormality, and retention in the study.

8. DATA COLLECTION

8.1 Participant charts

Each site will establish a chart for each participant enrolled in the study. Included in this chart will be a progress notes documenting each participant, checklists indicating completion of study procedures, and clinical study data. Additional detailed documentation requirements are contained in the HIVNET 015 (Explore) SSP manual.

8.2 Record Storage and Archive

All participant charts and DataFax forms must be stored in locked files in a secure area at the study site. Provided they contain no participant identifying information, the charts may be stored together with the DataFax forms. Otherwise, the charts and forms should be stored separately. Informed consent forms, release of information forms, and locator information must be kept in locked files in a secure area apart from DataFax forms and any other documents bearing Participant ID number (see also section 10.5.1).

Study sites will maintain all study documentation for at least five years after the completion of the study, unless otherwise specified by the PCT (see also Section 11.6).

9. STATISTICAL CONSIDERATIONS

9.1 Justification of sample size 1400 and 2000:

Primary Objectives 2.1.1 and 2.1.2: Age-related prevalence, incidence, and risk factors for anal human papillomavirus infection and anal cytologic abnormalities.

Table 1 shows margins of sampling error (i.e., the half-width of 95% confidence intervals) for baseline prevalence of HPV infection, expected to be in the range of 30-60%, and anal cytologic abnormalities, in the range of 5-20%, according to the age stratum of the sample and the prevalence.

Table 1 (a and b): Margins of sampling error in percentage points for baseline HPV and cytologic abnormalities prevalence, according to age stratum (rows) and the percent prevalence of the outcome (columns).

1a. Total sample size 2000

	5	10	20	30	40	50	60
<= 25	1.6	2.2	3.0	3.4	3.6	3.7	3.6
26-45	1.3	1.8	2.4	2.7	2.9	3.0	2.9
>45	3.5	4.8	6.4	7.3	7.8	8.0	7.8
Overall	1.0	1.3	1.8	2.0	2.1	2.2	2.1

1b. Total sample size 1400

	5	10	20	30	40	50	60
<= 25	1.9	2.7	3.5	4.1	4.3	4.4	4.3
26-45	1.5	2.1	2.8	3.2	3.5	3.5	3.5
>45	4.2	5.7	7.7	8.8	9.4	9.6	9.4
Overall	1.1	1.6	2.1	2.4	2.6	2.6	2.6

In the original overall sample, the margin of sampling error for HPV prevalence would have been approximately plus or minus 2.0-2.2 percentage points, depending on the actual prevalence; for men 25 or younger, the comparable margin would be 3.4-3.7 percentage points. Margins for the prevalence of cytologic abnormalities would be somewhat narrower, except in the small stratum of men over 45. In this table things don't look qualitatively worse.

In Table 2 a minimum odds-ratio with detectable 80% power is shown for the association of dichotomous risk factors with prevalence of these outcomes. In these computations the sample size is penalized by 10% to account for covariate adjustment.

Table 2 (a and b): Minimum detectable odds-ratios for the association of dichotomous exposures with prevalent HPV or cytologic abnormalities at

baseline according to the percent prevalence of exposure (rows), and the percent prevalence of the outcome (columns).

1a. Total sample size 2000

	5	10	20	30	40	50	60
10	2.4	1.9	1.7	1.6	1.6	1.6	1.6
30	1.9	1.6	1.4	1.4	1.3	1.3	1.4
50	1.8	1.6	1.4	1.3	1.3	1.3	1.3
70	2.1	1.7	1.5	1.4	1.3	1.3	1.3
90	3.1	2.5	1.8	1.7	1.5	1.6	1.6

1b. Total sample size 1400

	5	10	20	30	40	50	60
10	2.8	2.2	1.9	1.8	1.7	1.7	1.8
30	2.2	1.8	1.5	1.5	1.4	1.4	1.4
50	2.1	1.7	1.5	1.4	1.4	1.4	1.4
70	2.6	1.9	1.6	1.5	1.4	1.4	1.4
90	6.4	3.1	2.2	1.9	1.8	1.7	1.7

In most cases, minimum detectable odds-ratios with the sample of 2000 were considerably less than 2, and this remains the case. Minimum detectable associations with continuous predictors would be smaller.

Incidence of both cytologic abnormalities and HPV over the course of the 2 years is expected to be in the range of 5-20% of those at risk. Of the overall sample, 40-70% are expected to be at risk for incident HPV infection, while 10-50% -- those with prevalent or incident HPV infection -- may be at risk for progression to cytologic abnormalities. Table 3 shows margins of sampling error for cumulative incidence, given this range of subsample sizes.

Table 3 (a and b): Margins of sampling error for incident HPV and cytologic abnormalities, according to sample size of subset (rows) and percent incidence of outcome (columns).

3a. Total sample size 2000

	5	10	15	20
200	3.0	4.2	4.9	5.5
800	1.5	2.1	2.5	2.8
1000	1.4	1.9	2.2	2.5
1400	1.1	1.6	1.9	2.1

3b. Total sample size 1400

	5	10	15	20
140	3.6	5.0	5.9	6.6
560	1.8	2.5	3.0	3.3
700	1.6	2.2	2.6	3.0
980	1.4	1.9	2.2	2.5

Table 4 shows minimum detectable odds-ratios for the association of dichotomous predictors with incident HPV infection, under the original assumptions that 1000 study participants are at risk and that the HPV endpoint is ascertained for 85% of them. In the new version, 700 would be at risk instead of 1000.

Table 4 (a and b): Minimum detectable odds ratios for dichotomous predictors of inicident HPV, according to percent exposure prevalence (rows) and percent cumulative incidence (columns)

4a. Total 1000 at risk

	5	10	15	20
20	3.0	2.3	2.0	1.9
40	2.8	2.1	1.8	1.7
60	3.4	2.2	1.9	1.8
80	8.0	3.1	2.4	2.1

4b. Total 700 at risk

	5	10	15	20
20	3.7	2.7	2.3	2.1
40	3.5	2.4	2.1	1.9
60	5.0	2.7	2.2	2.0
80	NA	4.6	3.1	2.5

Minimum detectable odds-ratios would have been 2 or smaller in many situations, provided incidence is at least 10%. This is no longer the case, but things are not qualitatively worse. Again, minimum detectable associations with continuous predictors will be smaller.

The incidence of HIV infection was expected to be 1.55 per 100 person-years in the usual care arm of the trial, approximately 30% lower in the behavioral intervention arm, and, with remote follow-up, ascertained for 95% of the total cohort. Cumulative incidence in the two years of the trial was to have been about 2.6%. New estimates suggest that cumulative incidence may be higher, but since the HPV sample will have been recruited later than originally expected, this may be outweighed by shorter follow-up in for HPV ppts. Table 5 shows minimum detectable odds-ratios in the sample of 2000, for the association of prevalent HPV infection or anal cytologic abnormalities with incidence of HIV, according to the percent prevalence of the exposure (rows), and the percent cumulative incidence of HIV (columns). The second version of the table shows the same results for sample size 1400.

Table 5 (a and b): Minimum detectable odds-ratios for prevalent HPV or cytologic abnormalities as predictors of cumulative incidence of HIV, according to percent exposure prevalence (rows) and percent cumulative HIV incidence (columns).

5a. Total sample size 2000

	2	2.5	3
5	5.5	4.8	4.4
10	4.1	3.6	3.3

20	3.4	3.0	2.7
40	3.3	2.8	2.5
60	4.3	3.5	3.0

5b. Total sample size 1400

	2	2.5	3
5	7.2	6.2	5.5
10	5.2	4.5	4.0
20	4.3	3.7	3.3
40	4.4	3.6	3.2
60	7.7	5.1	4.1

9.2 Analysis Plan

Baseline and follow-up data will be entered on DataFax forms and faxed to the HIVNET Statistical Center (SC). The accuracy and internal consistency of the data will be assured by well-established HIVNET procedures. In preliminary analysis, bivariate relationships will be assessed using cross-tabulation, comparison of means and percentiles, and graphical tools. We will use pooled logistic regression (17,18) to examine predictors of loss-to-follow-up in the intervals between visits. In addition, if loss-to-follow-up exceeds 10%, we will use multiple imputation (19) to assess the potential for attrition bias.

Specific aims 1 and 2: The pooled adjacent violators algorithm will be used to analyze baseline current status data on HPV infection, both type-specific and overall, as well as cytologic abnormalities, giving a nonparametric estimate of age-specific prevalence. In the cross-sectional analysis of risk factors for prevalent HPV and cytologic abnormalities, independent risk factors including age, sexual practices, and substance use will be assessed using logistic regression.

Age-specific person-time incidence rates together with Poisson confidence intervals will be computed using the prospective data. If reversion to negative PCR results is observed, we will use the EM algorithm to take account of possible misclassification of baseline negatives as uninfected, given the number of negative assays before their first positive result, if any.

Since the time of incident HPV infections will be interval-censored between study visits, pooled logistic regression will be used to identify independent risk factors for acquisition of HPV infection among study participants who are uninfected at baseline. We will check for effects of treatment on incidence, mediation of any treatment effect by follow-up risk behaviors, modeled by time-dependent covariates, and interaction of treatment with other risk factors. To increase power, these models will include all at-risk study participants; however, interactions of age with other risk factors will be examined.

Analogous pooled logistic regression models will be used to identify risk factors for development of cytologic abnormalities. In the statistical justification we take account of expected false negative results in computing minimum detectable odds-

ratios. Predictors of interest for both incident HPV and development of cytologic abnormalities will include concurrent HSV-2 seroincidence and rectal gonorrhea, both as time-dependent covariates.

Specific aim 3: New HIV infections will be interval censored so that pooled logistic regression will again be used to assess the effect of HPV infection on risk of acquiring HIV. It will be important to distinguish between direct effects of anal HPV infection, which should only increase risk of acquiring HIV among men who continue to practice unprotected receptive anal intercourse, and indirect effects, in which HPV infection serves as a marker for past (and thus also current) HIV risk behaviors. Thus we will control for such risk behaviors, and test for interaction between HPV and report of unprotected receptive anal intercourse in the preceding 6-12 months. We will also examine the interaction of treatment with HPV infection, since treatment is designed to reduce unprotected anal intercourse.

For all regression analyses, model selection will be guided by a preliminary examination of bivariate relationships, by tests for goodness of fit, by biological and psychological plausibility, and by previously reported associations and interactions.

10. HUMAN SUBJECTS

10.1 Institutional Review

Prior to study implementation, the protocol, informed consent forms, participant education materials, data collection instruments, and other requested documents shall be approved by local Institutional Review Board (IRB) that complies with 45 CRF 46. Subsequently, all protocols must be re-reviewed at least annually. All protocol amendments must be approved by the IRB prior to implementation. The study site is responsible for preparation and submission of all documents and periodic reports required by the IRB.
As described in section 11.2, PCT approval will follow local IRB approval.

10.2 Informed consent

Written informed consent will be obtained from each participant prior to enrollment, and participants will be provided with a copy of the form. Each study site is responsible for developing an informed consent form for local use based on the template in Appendix 1 as well as obtaining PCT and IRB approval of the local form. As described in Section 11.2, the PCT will review all locally developed informed consent forms prior to and following local IRB approval of the forms, to ensure adherence to template and 45 CRF 46. The PCT then will issue a formal approval to the study site to initiate study operations. Documentation of any subsequent changes in the consent forms must also be provided to the local IRB and the PCT prior to use at the study site.

10.3 Study Risks

10.3.1 Health Risks

There are no risks involved in obtaining one additional anal swab, but this may cause some discomfort.

10.3.2 Psychosocial Risks

Subjects may become anxious if they learn that they have abnormal anal cytology as a result of participating in this study. Participation in research may involve loss of privacy. Research records will be handled as confidentially as possible within the law. All records will be coded and kept in locked files, so that only the study investigators have access to them. No individual identities will be used in any reports or publications resulting from this study.

10.3.3 Procedures for minimizing risks

To minimize the above risks that involve discomfort, we try to be as gentle as possible. We will make it clear to the subjects who have abnormal anal cytology results that we do not yet understand the clinical significance of these findings and that they should continue safe sex practices.
To minimize the risk of loss of confidentiality, all records will be coded and kept in locked files, so that only the study investigators have access to them. No individual identities will be used in any reports or publications resulting from this study.

10.3.4 Discussion of risk to benefit ratio in this study

There are no serious risks of participating in this study. The benefits of participating include being made aware of the presence of abnormal anal cytology. As above, we will make it clear to the subjects that we do not yet understand the clinical significance of these findings and that they should continue safe sex practices. Men with HSIL will be referred for anoscopy and therapy. Subjects will be contributing to our understanding of the risk factors for anal HPV infection and ASIL. We already know that this contribution will be beneficial because of the potential for progression to anal cancer. It may also be beneficial if our study shows that anal HPV infection and/or ASIL are also risk factors for HIV acquisition, since this might lead to new approaches to reducing new HIV infection.

10.4 Study Benefits

10.4.1 Information

During the study, participants will receive the most current information and counseling about HPV infection and anal cytology. There is no current treatment for HPV infection, however participants may elect to seek ablative therapy for condylomata. Although the subjects receive no direct benefits other than knowledge of the results of their anal cytology, the information may be of great value in assessing the role of anal HPV infection and associated disease for others in the future. If an association is found then the results may be of great importance to future efforts to prevent HIV infection.

10.4.2 Access to Care

Identification of undiagnosed HPV infection or anal cytologic abnormalities is unlikely to have important benefits for the health of the study participants.

10.4.3 Benefit to Humanity

This study will provide information about age-related prevalence and incidence of HPV infection and abnormal anal cytology in men at high risk of

HIV infection. It will also provide information on the seroconversion rate in this population and any correlation with HPV infection and/or anal dysplasia. This information may be useful when planning screening programs for intra anal dysplasia and may contribute to the development of behavioral and medical interventions for HPV infection. Determining if HPV infection and/or anal dysplasia place men at higher risk of HIV infection may provide useful knowledge for planning alternative HIV prevention strategies.

10.5 Confidentiality

10.5.1 Local Protections

All local study records will be kept in locked file cabinets in areas with limited access. All local databases will be secured with password protected access systems. All study data and specimens will be identified by a coded ID number only, and records containing participant name or other personal identifiers will be stored apart from coded ID records.

Locator information and any list linking Participant ID numbers to file names or other identifying information will be stored in a separate, locked file in an area with limited access. If participant ID's are entered into a computer database, this database must be password-protected and maintained in a directory separate from any study-specific data. File encryption is encouraged, but not required.

Study information will not be released without the written permission of the participant, except as necessary for monitoring by the NIAID, the PCT, the SC or CL.

10.5.2 Statistical and Coordinating Center (SC) Protections

The SC provides several layers of participant data security including physical, network and computer access security. Physical security of all SC computers and data is obtained through controlled and limited access to areas containing the SC network. Access to the building and floor requires a personal keycard. All computer servers are kept in locked offices. All network-wiring closets are locked and only accessible to designated personnel.

Network security is established using a "firewall" computer to protect participant data and other SC files from attack via the Internet connection. This computer restricts and tightly regulates all network traffic between our internal (secure) SC network and the rest of the Internet Access security is obtained through the use of network login ID's, passwords and files protections. Every user is required to have an individual login name and password. A user connects to any SC computer from a network outside the HIVNET SC (e.g., through a dial-up connection or the Internet) will be required to use a SecureID card. The SecureID card generates a new, six-digit number every sixty seconds. This, along with a Personal Identification Number (PIN) known only to the registered owner of the SecureID card, forms the user's password. This system provides for an extremely secure remote access to SC computing resources. In addition, the DataFax data

management system provides additional levels of security that go beyond the normal system security. Users are limited by login name and password to the level of access that they have to any part of the DataFax server computer they immediately fall under the DataFax security umbrella. Users must both be registered to their DataFax security level as well as their system security level.

10.5.3 Federal Protections

The PCT will apply for a federal Certificate of Confidentiality for this study upon receipt from all study sites documentation of local IRB approval. The Certificate will apply to all study sites. Once secured, the Certificate of Confidentiality provides that study staff may not be compelled to disclose study-related information by any Federal, State or local, civil, administrative, or other proceedings.

10.6 Remuneration

Study participants will not be compensated for their time and effort in this HPV study.

11. ADMINISTRATIVE PROCEEDURES

11.1 Study Coordination

The study site investigators (or designees) and other key staff will participate in training sessions prior to study start-up. Therefore, the protocol chairs, biostatistician, and PCT protocol specialist will participate in conference calls, as required, to discuss site operations, progress, and difficulties, and coordination.

11.2 Study Activation

Study sites will be approved to initiate implementation of this study according to PCT Standard Operating Procedures. Briefly, the following steps will be completed:

- PCT review/approval of site-specific informed consent form
- Local IRB review/approval of protocol, site-specific informed consent form, participant education materials, advertising and other recruitment materials, data collection instruments, and any other documents requested by the IRB
- PCT review/approval of site activation documents: Investigator Agreement, curriculum vitae of the Principal Investigator, documentation of IRB approval, IRB-approved site-specific informed consent form, and protocol signature page.

11.3 Study Monitoring

NIAID, the PCT, SC and CL will conduct a site visit to monitor adherence to human subjects and other regulations, adherence to the study protocol and procedure manuals, the quality of data collection at the study sites, and the accuracy of data submitted to the SC. A minimum of two sites will be completed per year. Study sites will allow HIVNET site visitors to inspect study facilities and documents, as well as observe the performance of study procedures.

11.4 Protocol Compliance

This study will be conducted in full compliance with the protocol. The protocol will not be amended without prior written approval by the Protocol Team and the PCT

Project Officer. Protocol amendments must be submitted to the local IRB and PCT for approval prior to implementing any amended procedures.

Study sites will be approved to initiate implementation of study amendments according to PCT standard operating procedures, similar to those outlined for study initiation in Section 11.2.

11.5 Local Protocols
N/A

11.6 Study Records
The study site will maintain all study records for at least five years after the completion of the study, unless otherwise specified by the PCT. Study records include all of the following: Administrative documentation including study approval and initiation documents, site specific standard operating procedures, all reports and correspondence relating to the study. Participant case histories including consent forms, locator forms, DataFax forms and all other documents containing information pertinent to the study participant. Additional detailed information on study documentation requirements is contained in the HIVNET 015 (Explore) SSP manual.

11.7 Use of Information and Publications
All information collected for this study will be assembled, reviewed, and presented and/or published with the policies contained in section 4 of the HIVMOP.

11.8 Signatures and Timetable

Study site:

_____

Projected starting

date:_____

Projected accrual completion

date:_____

Projected follow-up completion

date:_____

The following documents must be submitted to the PCT Protocol Specialist prior to the initiation of any study operations.

- HIVNET Investigator's Agreement
- Curriculum Vitae for Principal Investigators
- Documentation of IRB approval
- IRB-approved consent form
- Completed protocol signature page

I have read the protocol entitled "Anal HPV infection and neoplasia as risk factors for HIV seroconversion" and agree to conduct the study in accordance with its provisions.

_____

_____

Principal Investigator **Date**

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APPENDIX 4

How To Take An Anal Cytology Swab

The following video demonstrates the method used at the University of California, San Francisco Anal Neoplasia Study to take an anal cytology swab.

When arranging the appointment it is usual to ask the subject to refrain from using an enema or douche, or from having anything inserted into the anal canal in the 24 hours prior to having the cytology swab. The reason for this is to increase the cellular yield when the cytology swab is taken.

The subject has the procedure explained and questions are answered.

A tray is set up next to the examination table, upon which is placed a Dacron™ swab moistened in tap water. Dacron™ must be used and not cotton because cotton swabs do not release their cells into ThinPrep™ as efficiently as Dacron™. A jar of ThinPrep™ fixative solution is also opened in preparation for the swab.

The subject is then placed in the left lateral position with their buttocks at the edge of the examination table. The subject is then made as comfortable as possible. The examiners' non-dominant hand is used to part the buttocks, allowing an unrestricted view of the anal margin.

Holding the Dacron™ swab in the other hand, the swab is gently inserted with a twisting motion to ten centimeters or until the examiners fingers touch the anal margin. The swab is then slowly removed with lateral pressure (enough to bend the

swab) and a spiral motion to most efficiently sample the entire anal circumference over the next ten seconds.

After removal the swab must be immediately submerged in ThinPrep™ solution to prevent cellular air-drying artifact. The swab is then agitated and pressed against the container walls to remove as much cellular material as possible. There is no need for concern if fecal contamination is present. The ThinPrep™ bottle should be tightly closed.

The procedure is now complete.

APPENDIX 5

Anal HPV And Anal Cytology Questions And Answers

Q What is this study all about?

A This study is about human papillomavirus, also known as HPV, or the wart virus, and infections with HPV in the anus. The investigators in this study and others have done many earlier studies to show that HPV infection is very common in gay and bisexual men. There are two reasons anal HPV is important: first, HPV can cause anal cancer, which in HIV-negative gay men is about 4 times more common than cervical cancer in women. HPV can also cause anal warts. Anal cancer is often preceded by precursors, known as squamous intraepithelial lesions or SIL. We believe that detection and treatment of SIL may prevent development of anal cancer.

Second, we are concerned that anal HPV infection may increase the risk of acquiring HIV infection. Since HPV causes warts, and warts bleed easily, it is conceivable that having an anal wart and having receptive anal intercourse may lead to a higher risk of getting HIV infection. So this study is trying to answer three important questions:

1) How common is anal HPV infection in HIV-negative men at different ages and in different ethnic groups?

2) How common is anal SIL in HIV-negative men at different ages and in different ethnic groups

3) Is there an association between having anal HPV infection or SIL and an increased risk of getting HIV infection?

In this study, we will look for anal HPV using a technique called Hybrid Capture. We look for anal SIL using a technique called anal cytology. To participate in this study you will have one anal swab every 6 months that will be used to detect both anal HPV and cytology. You will be given the results of your cytology within 2 months of your visit. You will not be given the HPV results since we will not be performing the test until the end of the study. If your anal cytology is abnormal, you may be referred for another test call anoscopy but this is not part of the study.

Q. Can you explain exactly what is meant by anal cytology?

A. Anal cytology is a test that samples cells from the anal canal. The cells are then examined by a pathologist who can grade the cells according to certain characteristics and report them as normal, abnormal cells of undetermined significance (ASCUS), low grade squamous intraepithelial lesions (LSIL) which includes wart virus change, high grade squamous intraepithelial lesions (HSIL), and cancer. It is important to understand that the cytology report is an indication of the actual grade of anal disease but that the cytology may sometimes be negative even when a lesion is present. The cytology may also not correlate with the actual grade of disease, which is usually determined by performing a biopsy.

Q. What is ASCUS?

A. This is a diagnosis which is between normal and LSIL. It may have nothing to do with anal HPV infection or it may reflect the presence of LSIL or HSIL.

Q. My anal cytology came back as LSIL. What does that mean?

A. LSIL consists of mild cellular changes including warty change. It is not thought to be a pre-cancerous lesion or even to represent an increased risk of developing anal cancer. However, in some people LSIL can progress into HSIL, which, in a very small number of cases may develop into cancer.

Q. So what should I do?

A. There are currently no guidelines for the management of LSIL. However, we currently recommend that if the LSIL persists for more than one year, then an anoscopy and biopsy should be performed. This is not part of our study. The anoscopy involves a clinician inserting an anoscope into the anal canal so that a biopsy can be taken of the lesion under direct vision. As a biopsy is the most definitive method used to diagnose abnormal tissue, unlike cytology, this will make sure that there is no HSIL present. If the biopsy confirms HSIL then we usually recommend treating it.

Q. What happens if my next anal cytology is normal?

A. This means that the pathologist did not see any abnormal cells. However, as I have mentioned, the sampling technique is not 100 per cent accurate so follow-up cytologies are necessary to make sure that any abnormal areas were not missed. You will be having 4 cytology tests every 6 months as part of this study.

Q. What happens if my cytology shows HSIL?

A. HSIL on cytology means that a potentially pre-cancerous area may be present in your anal canal. Although not proven, it is thought that HSIL may progress to cancer so we would normally want to confirm this diagnosis with an anoscopy and biopsy. If the biopsy confirms HSIL, you have the options of treating the lesion or observing it with regular cytologies over time. Observation may mean having an anal swab taken every 6 months to a year, although again, the optimal time spacing has not yet been demonstrated. If you choose treatment, you should be aware that none of the available treatment options have been fully validated with regard to outcome and recurrence of HSIL.

The treatment options currently available include applying acid directly onto the lesion in a doctor's office. This method works best for small discrete lesions that are not circumferential. When the lesion is too large, surgery is usually performed. The surgery is similar to having an operation on a hemorrhoid. It is performed as an outpatient in the operating room and includes removal of the lesion with a scalpel or laser, destruction with an electric needle. All of these procedures have potential complications and significant post-operative pain which has proven difficult to control. New medical therapies to treat HSIL without performing surgery are currently in early experimental stages.

APPENDIX 6

MAJOR ARTICLE

Age-Specific Prevalence of Anal Human Papillomavirus Infection in HIV-Negative Sexually Active Men Who Have Sex with Men: The EXPLORE Study

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Background. In the United States, anal cancer in men who have sex with men (MSM) is more common than cervical cancer in women. Human papillomavirus (HPV) is causally linked to the development of anal and cervical cancer. In women, cervical HPV infection peaks early and decreases after the age of 30. Little is known about the age-specific prevalence of anal HPV infection in human immunodeficiency virus (HIV)–negative MSM.

Methods. We studied the prevalence and determinants of anal HPV infection in 1218 HIV-negative MSM, 18–89 years old, who were recruited from 4 US cities. We assessed anal HPV infection status by polymerase chain reaction.

Results. HPV DNA was found in the anal canal of 57% of study participants. The prevalence of anal HPV infection did not change with age or geographic location. Anal HPV infection was independently associated with receptive anal intercourse (odds ratio [OR], 2.0; $P < .0001$) during the preceding 6 months and with >5 sex partners during the preceding 6 months (OR, 1.5; $P < .0001$).

Conclusions. Urban, HIV-negative MSM have a stable, high prevalence of anal HPV infection across all age groups. These results differ substantially from the epidemiologic profile of cervical HPV infection in women. This may reflect differences between these populations with respect to the number of new sex partners after the age of 30 and may explain the high incidence of anal cancer in MSM.

Anal cancer is increasing in incidence in women and men in the general population [1, 2]. Such subpopulations as men who have sex with men (MSM), HIV-positive women and men, transplant recipients, and women with cervical squamous intraepithelial lesions are at an even higher risk than the general population [3, 4]. Before the HIV epidemic, US MSM were estimated to have an incidence of anal cancer of up to 35 cases/100,000 person-years (py)—similar to the incidence of cervical cancer in US women before the introduction of cervical cytology testing [5].

Human papillomavirus (HPV) is one of the most common sexually transmitted infections. The causal link between HPV infection and cervical cancer has been well established [6]. HPV is also believed to be

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necessary for the development of anal cancer [4, 7] and its putative precursor lesion, anal intraepithelial neoplasia (also known as "anal squamous intraepithelial lesions" [ASILs]).

Although the age-specific prevalence of cervical HPV infection has been well described for women [8, 9], no studies have examined the age-specific prevalence of anal HPV infection in HIV-negative MSM. In women, the youngest sexually active age groups have a disproportionately high prevalence of HPV infection. The prevalence of cervical HPV infection decreases sharply in women after the age of 30, leaving only a fraction of women with persistent HPV infection; these women are thought to have the highest risk for the development of invasive cervical cancer [10]. To date, most of the published data on anal HPV infection were obtained from studies that were conducted in either San Francisco, CA, or Seattle, WA, and that were of HIV-positive MSM who spanned a narrow age range. However, the majority of MSM are HIV negative. To better understand the natural history of HPV infection and the potential effect of anal HPV infection on HIV-negative MSM, we here report the results of the first study investigating anal HPV infection in a geographically diverse population of HIV-negative MSM who span a wide age range.

PARTICIPANTS, MATERIALS, AND METHODS

Study population. Study participants were recruited from sites in 4 cities (Boston, MA; Denver, CO; New York, NY; and San Francisco, CA). All were participants of the HIV Prevention Trials Network EXPLORE study, a randomized clinical trial of the efficacy of a behavioral intervention to reduce the risk of HIV acquisition in sexually active HIV-negative MSM [11]. Males were eligible for the EXPLORE study if they were HIV negative at baseline, were ≥16 years old, and reported having receptive or insertive anal intercourse with ≥1 man during the preceding year. Recruitment strategies varied by city but included advertising and outreach at bars, bathhouses, clubs (including sex and health clubs), and video arcades. Referrals were also obtained via other cohort studies, current study participants, community agencies and clinics, the Internet, mailings, and a recruitment video. Baseline (study entry) visits for the EXPLORE study occurred from January 1999 to February 2001, with follow-up evaluations at 6-month intervals. Participants at the 4 sites were offered enrollment into the HPV substudy after the month-12 EXPLORE study visit. Between January 2001 and October 2002, 1409 men were enrolled into the HPV substudy. Of these, 1218 men contributed interpretable HPV data (β-globin positive). At enrollment, each participant provided written, informed consent. The present study was conducted with the approval of the institutional review boards of each participating institution. The human-experimentation guidelines of the US Department of Health and Human Services and those of the participating institutions were followed in the conduct of this clinical research.

Data collection. As part of the EXPLORE study, each participant reported drug use and sexual behavior using audio computer-assisted self-interview (ACASI) technology. Recorded questions were administered by audio or computer screen, and answers were inputted by keyboard. ACASI has been shown to increase the likelihood that such sensitive behaviors as having unprotected anal intercourse are reported, compared with the likelihood when interviewer-administered questionnaires are used [12].

Anal sample collection and HPV testing. Trained personnel at each site collected anal samples by rotating a water-moistened Dacron swab in the anal canal, without direct visualization. The swab was then agitated vigorously in 20 mL of a methanol-based fixative (PreservCyt; Cytyc) for HPV DNA testing by polymerase chain reaction (PCR).

To prepare samples, the PreservCyt solution was gently swirled to suspend cells. For each sample, 1.5 mL of the solution was transferred to a labeled microfuge tube by use of a transfer pipette. The tubes were spun at 16 *g* for 15 min, were decanted, and were dried either overnight or in a 65°C hot block for 1 h. The pellets were suspended in 100 μL of sample transport medium (Digene) and 2 μL of 10 mg/mL proteinase K (PK; Boehringer Mannheim). The samples were vortexed and digested in a water bath for 1 h at 56°C; were heated for 10 min at 95°C, to inactivate PK; and were frozen until use. For amplification, 5 μL of each sample was used, and PCR was performed according to a standard 40-cycle protocol [13]. PCR products from positive samples were typed by dot-blot hybridization, with 39 type-specific probes.

Statistical analyses. Age-specific prevalence of HPV was estimated by use of sample proportions. Independent predictors of anal HPV infection were identified by use of logistic regression. Predictors that were significant at $P < .15$ in both the univariate and multivariate analysis were retained in the final multivariate models. Analyses were conducted by use of Stata software (version 8.0; Stata Corporation).

RESULTS

The median age of participants in the present study was 37 years (table 1). Sixty-seven percent had an annual income of ≥$30,000, and 72% had at least an undergraduate degree. Seventy-eight percent were white, 14% were Latino, 6% were African American, and 3% were Asian. The median age at first receptive anal intercourse was 20 years. Forty-nine percent were current smokers or had smoked ≥100 cigarettes in the past. Participants reported a median of 8 sex partners during the preceding 6 months, and 77% reported having receptive anal intercourse during the preceding 6 months. Eight percent reported a history of injection drug use. There was no statistical

Table 1. Selected demographic and behavioral characteristics of 1409 participants at enrollment.

Characteristic	Value
Age, median (IQR), years	37 (31–43)
Education, undergraduate degree or more	72
Income, >$30,000 annually	67
Ethnicity	
Latino	14
Non-Latino	86
Race	
Asian	3
African American	6
White	78
Mixed	13
Age at first anal receptive intercourse, median (IQR), years	20 (17–24)
No. of male sex partners during the preceding 6 months, median (IQR)	8 (4–20)
Receptive anal intercourse during the preceding 6 months	77
Condom use during the preceding 6 months, always	38
Current smoker	22
Injection drug use during the preceding 6 months	8

NOTE. Data are percentage of participants, unless otherwise noted. IQR, interquartile range.

difference between the HPV substudy participants and the entire EXPLORE cohort with respect to age, ethnicity or race, income, sexual behavior, and illicit drug use.

Age-specific prevalence of anal HPV infection. The overall prevalence of anal HPV infection was 57%. Figure 1 and table 2 show the age-specific prevalence of HPV infection in participants. The prevalence of HPV infection was similar across all age groups. The most common HPV type detected in this study population was HPV-16, identified in 12% of participants. The prevalence of all high-risk HPV types (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68, and 73) was 26% and was similar across all age groups. The prevalence of all low-risk HPV types (6, 11, 53–55, 66, Pap 155, and Pap 291) was also 26%. HPV-positive participants were infected with a mean of 1.8 HPV types (range, 1–10 HPV types); this value, too, was similar across all age groups. Forty-five percent of HPV-positive participants were infected with >1 HPV type. Figure 2 shows the distribution of the number of HPV types infecting each participant, by age group. Figure 3 shows the age-specific distribution of the 5 most common HPV types detected in the anal canals of participants (16, 6, 11, 53, and 18). In logistic models that adjusted for variables that potentially confound the association between age and type, the prevalence of these HPV types and the next 5 most common HPV types (31, Pap 155, 33, 61, and 66) was similar across all age groups.

The prevalence of anal HPV infection was similar across study sites. Sixty-one percent of participants in San Francisco, 57% of participants in Boston, 60% of participants in New York, and 49% of participants in Denver were found to have anal HPV infection.

Risk factors for anal HPV infection. In a multivariate logistic model of predictors of having any anal HPV infection, there was strong evidence for an effect of a history of receptive anal intercourse and of the number of male sex partners during the preceding 6 months (table 3). The following variables were not significant in univariate analysis ($P > .20$): age; smoking; age at first receptive anal intercourse; ethnicity or race; education; employment status; condom use; use of alcohol, marijuana, crack, ecstasy, or injection drugs; and recent history or diagnosis of chlamydia, gonorrhea, syphilis, or genital sores.

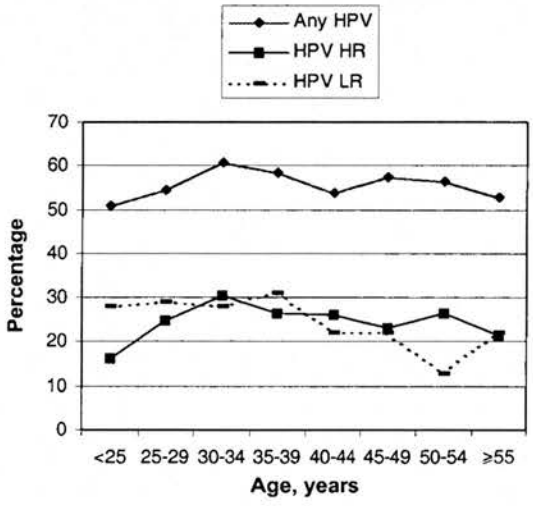

Figure 1. Prevalence of human papillomavirus (HPV) DNA in the anal canals of HIV-negative men who have sex with men, by age group and by cancer-associated risk type. High-risk (HR) types include 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68, and 73; low-risk (LR) types include 6, 11, 53–55, 66, Pap 155, and Pap 291.

Table 2. Prevalence of human papillomavirus (HPV) DNA in the anal canals of HIV-negative men who have sex with men, by age and cancer-associated risk type.

Category	<25 years	25–29 years	30–34 years	35–39 years	40–44 years	45–49 years	50–54 years	≥50 years	Total[a]
Any HPV type	50/98 (51)	91/167 (55)	165/271 (61)	160/273 (59)	97/180 (54)	72/125 (58)	30/53 (57)	27/51 (53)	692/1218 (57)
HPV HR type	16/98 (16)	41/167 (25)	82/271 (30)	72/273 (26)	47/180 (26)	29/125 (23)	14/53 (26)	11/51 (22)	312/1218 (22)
HPV LR type	27/98 (28)	48/167 (29)	77/271 (28)	84/273 (31)	40/180 (22)	28/125 (22)	7/53 (13)	11/51 (22)	322/1218 (22)

NOTE. Data are proportion (%) of study population. High-risk (HR) types include 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68, and 73; low-risk (LR) types include 6, 11, 53–55, 66, Pap 155, and Pap 291.

[a] Of 1409 baseline samples, 191 were uninterpretable by polymerase chain reaction (β-globin negative).

The following variables were included but were not significant in multivariate proportional-hazards analysis: age, smoking, age at first receptive anal intercourse, and income. In a multivariate logistic model of predictors of infection with any high-risk HPV type, there also was strong evidence for an effect of a history of receptive anal intercourse during the preceding 6 months (odds ratio [OR], 2.1 [95% confidence interval {CI}, 1.4–3.2]; $P < .0001$) and moderate evidence for an effect of number of male sex partners >5 during the preceding 6 months (OR, 1.2 [95% CI, 1.0–1.5]; $P = .06$), with age, smoking, and age at first receptive anal intercourse controlled for.

DISCUSSION

This is the first study to investigate the age-specific prevalence of anal HPV infection in HIV-negative MSM. The striking finding of the present study is that urban HIV-negative MSM have high rates of anal HPV infection across all age groups. Using PCR testing, we found that 57% of the HIV-negative MSM in the present study were HPV positive and that 26% were infected with a high-risk HPV type. Therefore, a high proportion of HIV-negative MSM may be at risk for developing anal cancer.

Multiple studies have shown that, in women, cervical HPV infection is strongly related to age [8, 9]. Most cervical HPV infections are believed to be self-limited. A small proportion of women have persistent HPV infections, leading to a peak in precancerous cervical cytologic abnormalities during the age range of the late 20s [8]. In addition, there is a second, smaller peak in the prevalence of HPV infection in women after the age of 55 [5]. It is speculated that this second peak is due to new exposures in older women or to age-related attenuation of immune responses. In contrast, the age-specific prevalence of anal HPV infection in the HIV-negative MSM in the present study was similar across all age groups.

The difference between the age-related prevalence of HPV infection of the cervix in women and of the anus in men may be explained by several factors. Despite a common embryologic origin and transformation zone histology [14], there may be organ-specific differences and unique hormonal environments that account for this disparity. HPV may persist longer in the anus, for example, or hormone-related changes in the transformation zone in women may make them more susceptible to HPV infection at particular times. Another explanation for the disparity may be differences in sexual behavior between the HIV-negative MSM in the present study and women in the general population. Our study participants had more new sex partners than has been reported by most women >30 years old

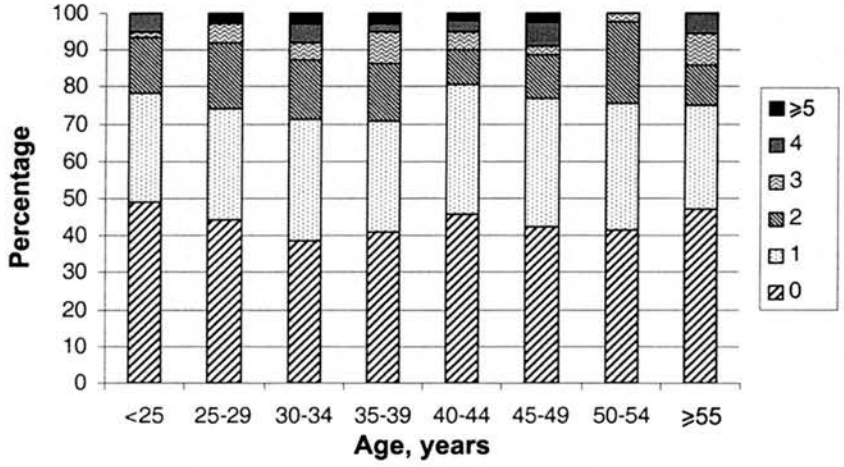

Figure 2. Distribution of the number of human papillomavirus (HPV) types infecting each participant in the study population of HIV-negative men who have sex with men, by age group.

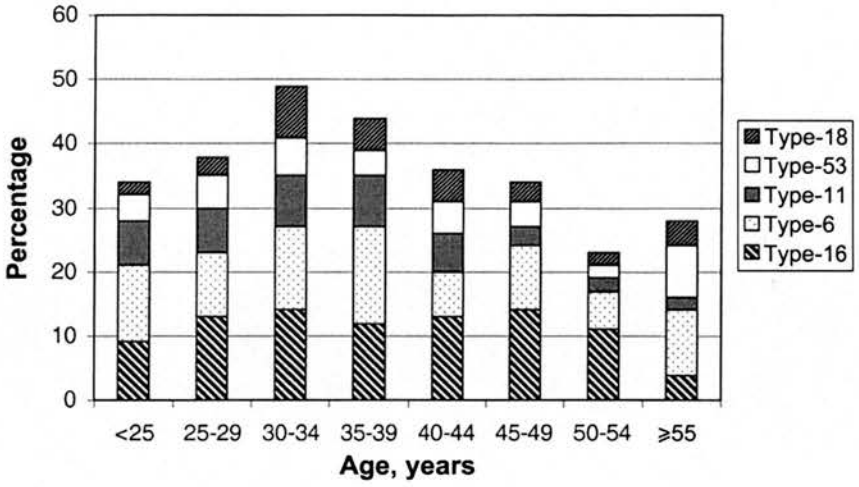

Figure 3. Distribution of human papillomavirus (HPV) types in HIV-negative men who have sex with men. The 5 most common HPV types (16, 6, 11, 53, and 18) detected in the anal canals of the study population are shown.

[15]. New exposures best explains the high prevalence after the age of 30 years, because the main risk factor for anal HPV infection was the number of male sex partners during the preceding 6 months. However, because the overall prevalence of HPV and the mean number of HPV types did not increase with age, our data also suggest that most newly acquired HPV infections are transient—or that, if they are persistent, they are suppressed to levels that are too low to be detectable by PCR.

If lifelong type-specific immunity is gained when an HPV type is cleared, then we would expect that the spectrum of HPV types detected in the present study population would have varied among the different age groups. However, this was not observed (figure 3). We propose that lifelong immunity does not persist and that individuals exposed to a given HPV type might be susceptible to infection, at least transiently, even if previously infected with that type. Prospective studies of MSM will be needed to definitively answer this question.

The prevalence of HPV infection was high in our study population, a finding similar to those of previous studies [16]. A wide array of HPV types was identified, and multiple infections were common. HPV-16, a high-risk HPV type strongly linked to invasive cervical and anal cancer, was the most frequent type identified, another finding similar to those of previous studies [17]. Risk factors for any anal HPV infection and high-risk HPV infection that were identified in our multivariate analysis included receptive anal intercourse and the number of male

Table 3. Risk of infection with any human papillomavirus (HPV) type.

Risk factor	Proportion (%) of MSM infected with HPV	HPV infected vs. uninfected			
		Crude		Adjusted	
		OR (95% CI)	*P*	OR (95% CI)	*P*
Overall	692/1218 (56.8)				
Receptive anal intercourse during the preceding 6 months			<.0001		<.0001
No	114/274 (41.2)	1.0		1.0	
Yes	579/944 (61.3)	2.3 (1.7–3.0)		2.0 (1.5–2.8)	
Use of cocaine during the preceding 6 months			.01		.42
No	555/1006 (55.1)	1.0		1.0	
Yes	137/211 (64.9)	1.5 (1.1–2.0)		1.2 (0.8–1.7)	
Use of "poppers" during the preceding 6 months			.02		.78
No	416/767 (54.2)	1.0		1.0	
Yes	276/448 (61.6)	1.3 (1.0–1.7)		1.0 (0.7–1.3)	
No. of male sex partners during the preceding 6 months			<.0001[a]		<.0001[a]
≤5 partners	232/464 (50.0)	1.0		1.0	
6–30 partners	360/606 (59.4)	1.5 (1.1–1.8)		1.4 (1.1–1.9)	
>30 partners	99/147 (67.4)	2.1 (1.4–3.2)		2.3 (1.5–3.6)	

NOTE. CI, confidence interval; MSM, men who have sex with men; OR, odds ratio.

[a] For linear trend.

sex partners during the preceding 6 months. These findings are also consistent with those of previous studies [13].

Two of the strengths of the present study are its size and that it is the first multicity study of anal HPV infection. One potential limitation of the present study is that it is not clear whether our results can be generalized to some groups of MSM. EXPLORE participants were HIV-negative MSM who had had anal intercourse during the year preceding study entry. Although we do not know whether these results can be generalized to MSM in rural areas, we do believe that these results can be generalized to MSM in urban areas, where a high proportion of MSM live. Data from the population-based Urban Men's Health Study (UMHS) [18], conducted in 4 cities, suggest that our cohort is highly representative of urban MSM with respect to age at first anal intercourse, history of any anal sex, and anal sex during the preceding year. The UMHS has shown that 91% of all MSM in San Francisco, Chicago, New York, and Los Angeles had at least 1 sex partner during the preceding year. We considered MSM >45 years old to compose the group that was most likely to be the least comparable to the EXPLORE cohort; however, even this group in the UMHS was similar to the EXPLORE cohort. Among MSM >45 years old in the UMHS, 48% had >1 sex partner during the preceding year, and 74% had at least 1 sex partner during the preceding year. At a minimum, the findings of the present study can be generalized to 50%–75% of urban MSM. Therefore, we strongly believe that the present study has broad public-health implications. Other recent cross-sectional surveys of MSM in San Francisco [19], London [20], and other cities report comparable sexual behaviors in respondents.

In the present study, the proportion of β-globin–negative samples was moderately high (>10%). This may reflect the inexperience of the study personnel who collected the samples using Dacron swabs, rather than any inherent defect in the Dacron material. In future prospective analyses, analysis of the proportion of insufficient anal HPV samples stratified by provider experience will help answer this question. Finally, although the MSM in the present study were HIV negative at baseline, there is a chance that a small proportion of them had seroconverted to HIV positive by the time of enrollment into the HPV substudy. The Vaccine Preparedness Study, which had a population similar to ours, reported an HIV-seroconversion rate of 1.5 seroconversions/100 py [21]. This rate would affect our anal HPV and ASIL prevalence estimates only slightly, because of the relatively small numbers involved.

Given the results of the present study, assumptions about extrapolating data from cervical disease in women to anal HPV infection in HIV-negative MSM must be questioned. Our data suggest that anal HPV infection in HIV-negative MSM has a unique epidemiologic profile that must be carefully explored in future studies, particularly in light of promising new HPV therapeutic and prophylactic vaccines [22].

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APPENDIX 7

Age-Related Prevalence of Anal Cancer Precursors in Homosexual Men: The EXPLORE Study

Peter V. Chin-Hong, Eric Vittinghoff, Ross D. Cranston, Lynette Browne, Susan Buchbinder, Grant Colfax, Maria Da Costa, Teresa Darragh, Dana Jones Benet, Franklyn Judson, Beryl Koblin, Kenneth H. Mayer, Joel M. Palefsky

Background: **Infection with human papillomavirus (HPV) is causally linked to the development of anal and cervical cancer. In the United States, the incidence of anal cancer among men who have sex with men (MSM) is higher than the incidence of cervical cancer among women. Anal squamous intraepithelial lesions (ASILs) are anal cancer precursors comprising low-grade squamous intraepithelial lesions (LSILs) and high-grade squamous intraepithelial lesions (HSILs). The prevalence of cervical cancer precursor lesions peaks at around 30 years of age. The age-related prevalence of ASILs in HIV-negative MSM is unknown.** ***Methods:*** **We conducted a cross-sectional analysis of the prevalence and determinants of ASILs in 1262 HIV-negative MSM aged 18–89 years recruited from four U.S. cities. Anal cytology and behavioral data were obtained. Anal HPV infection status was assessed by polymerase chain reaction. Independent predictors of ASILs were identified using logistic regression. All statistical tests were two-sided.** ***Results:*** **The prevalences of LSILs and HSILs were 15% and 5%, respectively, and did not change with age. In a multivariable analysis, the risk of LSILs was associated with having more than five male receptive anal sex partners ($P = .03$), any use of poppers (alkyl nitrites) in the previous 6 months [odds ratio (OR) = 1.6, 95% confidence interval (CI) = 1.1 to 2.5; $P = .03$] or use of injection drugs two or more times per month during the previous 6 months [OR = 19, 95% CI = 1.3 to 277; $P = .03$], older age at first receptive anal intercourse ($P = .004$), and infection with a greater number of HPV types ($P < .001$ for linear trend). The risk of HSILs was associated with any anal HPV infection (OR = 3.2, 95% CI = 1.1 to 9.4; $P = .039$) and infection with an increasing number of HPV types ($P < .001$ for linear trend).** ***Conclusions:*** **Sexually active HIV-negative MSM in all age groups have a high prevalence of ASILs, possibly reflecting their ongoing sexual exposure to HPV. [J Natl Cancer Inst 2005;97:896–905]**

Before the human immunodeficiency virus/acquired immunodeficiency syndrome (HIV/AIDS) epidemic, it was estimated that, in the United States, the incidence of anal cancer in men who have sex with men (MSM) is higher than the incidence of cervical cancer in women *(1,2)*. Current data indicate that the incidence of anal cancer in men has continued to increase *(3)*. Infection with human papillomavirus (HPV) is causally linked to the development of anal and cervical cancers and their associated precancerous lesions. Anal squamous intraepithelial lesions (ASILs) are the putative precursors of anal cancer, just as cervical squamous intraepithelial lesions (CSILs) are the precursors of cervical cancer *(4–8)*. ASILs range from low-grade squamous intraepithelial lesions (LSILs) to high-grade squamous intraepithelial lesions (HSILs). We believe that HSILs are the true invasive anal cancer precursors. LSILs are not believed to be a direct precursor to anal cancer but may precede the later development of HSILs *(7)*.

Several studies have shown that the detection of cervical cytologic abnormalities (i.e., LSILs and HSILs) is strongly associated with age. Using population-based sampling, Herrero et al. *(9,10)* demonstrated that the prevalence of LSILs in the cervix peaked at 5.2% among women younger than 25 years, decreased sharply to 2.7% by ages 25–34 years, and then continued to decrease to 0.4% among women older than 65 years. The prevalence of HSILs in the cervix peaked later, at ages 25–34 years, and was followed by a less pronounced decline at ages 35–44 years and a smaller increase in prevalence among women older than 65 years old compared with the increase that was observed at ages 25–34 years. It has been estimated that cervical HSILs, if undetected, can progress to invasive cervical cancer in 9–10 years *(11)*.

Knowledge of the age-related prevalence and natural history of CSILs has guided the development of screening strategies for cervical cancer, in which women older than 30 years who have had no previously detected lesion may undergo cytology screening at longer than annual intervals because they have a relatively low likelihood of developing a cervical lesion de novo *(12,13)*. Although several studies have examined the age-associated distribution of CSILs in women *(9,14)*, and one study has investigated the age-associated prevalence of anal HPV infection in MSM *(15)*, no studies have reported the age-associated prevalence of ASILs in MSM or in other populations at high risk for anal cancer. Two studies of ASILs in MSM reported the prevalence, incidence, and risk factors for ASILs for a narrow age spectrum of men living in Seattle *(16)* or San Francisco *(17)* only. As is the case for CSILs, understanding how age affects the prevalence of ASILs may have implications for anal cytology screening recommendations.

The objectives of this study were to describe the age-related prevalence and risk factors for ASILs in MSM. We also compared the prevalence of ASILs among MSM in four U.S. cities.

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See "Notes" following "References."

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We report results of the first study to describe ASILs in a geographically diverse population of HIV-negative MSM spanning a wide age range.

Subjects and Methods

Study Population

We enrolled men who were concurrently enrolled in four of six EXPLORE trial study cities (Boston, Denver, New York, and San Francisco). The EXPLORE trial is a randomized clinical trial of the efficacy of a behavioral intervention to reduce the risk of HIV acquisition among sexually active HIV-negative MSM *(18–20)*. Men were eligible for the EXPLORE trial if, at baseline, they were HIV-negative, were 16 years of age or older, and reported having had receptive or insertive anal sex with one or more men during the previous year. Men who had been involved in seroconcordant, monogamous relationships with a male partner for 2 or more years were excluded from the EXPLORE trial. Recruitment strategies for the EXPLORE trial varied slightly among study sites and included advertising and outreach at clubs, bars, bathhouses, sex clubs, health clubs, and video arcades. EXPLORE baseline visits occurred from January 28, 1999, to February 7, 2001, and subsequent visits were made at 6-month intervals. Enrollment in the HPV substudy was offered to EXPLORE participants in four of six cities after the month 12 EXPLORE study visit. From January 2, 2001 to October 23, 2002, 1409 men were enrolled in the HPV substudy. At enrollment, each participant provided written informed consent. This study was conducted with the approval of the institutional review boards of each participating center (the University of California at San Francisco; the Department of Public Health at San Francisco, CA and Denver, CO; the New York Blood Center; and the Fenway Community Health Center).

Data Collection

The EXPLORE trial collected information on participants' reported drug use and sexual behaviors with the use of Audio Computer-Assisted Self-Interview (ACASI) technology *(21)* that enabled study participants to either hear or read the questions and enter their answers on a keyboard. Both English and Spanish versions of the study questionnaire were available. Compared with interviewer-administered questionnaires, ACASI has been shown to increase the likelihood that sensitive behaviors, such as unprotected anal intercourse, are accurately reported *(21)*. We obtained additional data on sexual history and smoking for the HPV substudy by having each participant complete a questionnaire which they then sealed in an envelope after completion for collection. Questionnaires were batched and returned to us by study coordinators.

Anal sample collection and cytology. Trained personnel at each study site collected anal specimens from each study participant by rotating a water-moistened Dacron swab in the anal canal without direct visualization. The swab was then agitated vigorously in a methanol-based fixative (PreservCyt solution; Cytyc Corporation, Boxborough, MA). The resulting solution was then stored at room temperature and later used for the preparation of thin-layer slides for cytology as well as for the detection of HPV DNA by the polymerase chain reaction (PCR). Anal cytology was evaluated by a single pathologist (T. Darragh) who had no knowledge of the clinical status of the participants, their questionnaire or ACASI responses, or other test results. Anal cytology specimens were classified as normal, atypical squamous cells (ASCs) comprising ASCs of undetermined significance and ASCs that cannot exclude HSILs), LSILs, or HSIL, using the 2001 Bethesda System criteria that are used to evaluate cervical cytology *(22)*.

HPV DNA testing. To prepare DNA from the anal specimens, we gently swirled the PreservCyt solutions containing the anal sample to suspend the cells, removed 1.5 mL of the solution to labeled microfuge tubes, and centrifuged the tubes at 16*g* for 15 minutes. The supernatants were decanted, and pelleted material was dried overnight at room temperature or for 1 hour at 65 °C. The pellets were resuspended in 100 μL Sample Transport Medium (Digene, Silver Spring, MD) and 200 μg/mL proteinase K (Boehringer Mannheim, Indianapolis, IN), mixed by vortexing, incubated for 1 hour at 56 °C in a water bath, heated for 10 minutes at 95 °C for to inactivate the proteinase K, and frozen at −20 °C.

We used 5 μL of each sample to detect the presence of one or more HPV types by PCR amplification using a standard 40-cycle protocol, as previously described *(23)*. PCR products from positive samples were typed by dot-blot hybridization with 39 type-specific probes. We classified HPV types as high risk (HPV types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68, or 73) or low risk (HPV types 6, 11, 53, 54, 55, 66, Pap 155, or Pap 291) based on the strength of the association of specific HPV types to invasive anogenital cancer *(24,25)*.

Statistical Analysis

We used sample proportions to estimate the age-specific prevalence of LSILs and HSILs. Independent determinants of ASILs were identified using logistic regression methods. Factors that were statistically significantly associated with ASILs at $P<.15$ in univariate analyses, as well as those that were identified as being important in previous studies, were retained in the final multivariable models. We used a polytomous logistic regression model to estimate and compare associations with risk factors that distinguished between participants with normal cytology (the referent group) and participants with LSILs and HSILs. The analysis was carried out using Stata software (version 8.0; Stata Corporation, College Station, TX). All statistical tests were two-sided.

Results

Characteristics of Study Participants at Baseline

Of 1409 participants recruited for this study, samples from 1262 men were sufficient for cytologic analysis at baseline. Participants had a median age of 37 years (interquartile range [IQR] = 31–43 years) (Table 1). The median age of participants at first receptive anal intercourse was 20 years (IQR = 17–24 years). Study participants reported having a median of eight sex partners (IQR = 4–20) and three receptive anal sex partners (IQR = 0–11) during the previous 6 months. Men younger than 35 years reported a median of eight sex partners (IQR = 4–16 partners), men at least 35 years old but younger than 50 years also reported a median of

Table 1. Characteristics of the study population*

Characteristic	Value
Median age, y (IQR)	37 (31–43)
Education, college degree, %	72
Annual income, ≥ $30 000.00, %	67
Race/ethnicity, %	
Asian	3
African American	5
Hispanic	14
White	67
Mixed	11
Median age at first anal receptive intercourse, y (IQR)	20 (17–24)
Any female sexual partners, %†	2
No. of male sexual partners, %†	
<2	8
2–5	30
>5	62
No. male receptive anal intercourse sexual partners, %†	
<2	32
2–5	24
>5	44
Condom use, always, %†	38
Smoking status, %	
Never	52
Former	26
Current smoker, <1 pack/day	11
Current smoker, ≥1 pack/day	11
Injection drug use, %†	8
Diagnosed with genital warts, %†	4

*IQR = interquartile range.
†During the previous 6 months.

eight sex partners (IQR = 4–20 partners), and men 50 years of age or older reported a median of 10 partners (IQR = 3–24 partners) during the previous 6 months. Condoms were always used by 38% of the study participants during the previous 6 months. Participants in San Francisco, New York, Boston, and Denver were similar with respect to median age, other sociodemographic factors, and the median number of receptive anal sex partners they reported having in the previous 6 months (data not shown). There was no statistically significant difference between HPV substudy participants and all enrollees in the EXPLORE trial or between HPV substudy participants with sufficient cytologic samples and those with insufficient samples with respect to age, race, income, sexual behavior, or illicit drug use (data not shown).

Prevalence of ASILs by Age and Geographic Location

The overall prevalence of any abnormal cytology (ASCs, LSILs, or HSILs) in this population was 32% and was similar for all age groups (i.e., a test of trend was not statistically significant). The overall prevalence of ASILs (either LSILs or HSILs) was 20%, and the prevalences of LSILs and HSILs were 15% and 5%, respectively. The prevalences of LSILs and HSILs were similar for all age groups (Figure 1).

The prevalence of ASILs was also similar for all study sites (Figure 2). Cytologic examination revealed that 23% of participants in San Francisco, 19% of participants in New York, 21% of participants in Boston, and 17% of participants in Denver had ASILs ($P = .23$ for any differences between cities). Study participants in San Francisco had the highest prevalence of HSILs (8%), followed by those in New York (5%), Boston (4%), and Denver (4%) ($P = .051$ for any differences between cities). The prevalences of LSILs and ASCs were similar when stratified by city.

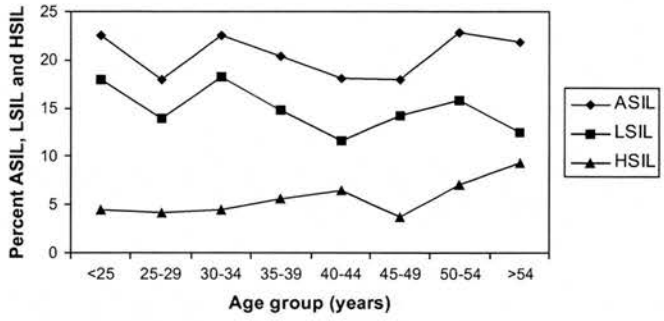

Fig. 1. Prevalence of anal squamous intraepithelial lesions (ASIL) by age group. LSIL = low-grade squamous intraepithelial lesion; HSIL = high-grade squamous intraepithelial lesion.

Risk Factors for ASILs

Variables that were statistically significantly associated with the risk of ASILs in unadjusted analyses included higher number of male receptive anal sex partners in the previous 6 months ($P<.001$ for linear trend), any use of poppers (alkyl nitrites) compared with no use in the previous 6 months (OR = 1.6, 95% CI = 1.2 to 2.1, $P = .002$), use of injection drugs two or more times per month compared with no use in the previous 6 months (OR = 6.7, 95% CI = 1.7 to 27, $P = .007$), anal HPV infection (OR = 7.5, 95% CI = 4.9 to 11, $P<.001$), and anal HPV infection with increasing numbers of HPV types ($P<.001$ for linear trend) (Table 2). In unadjusted analyses, the risk of ASILs was also statistically significantly associated with infection with high-risk HPV types only (OR = 5.1, 95% CI = 3.1 to 8.4, $P<.001$), infection with low-risk HPV types only (OR = 9.6, 95% CI = 6.0 to 15, $P<.001$), and infection with both high-risk and low-risk HPV types (OR = 21, 95% CI = 12 to 35, $P<.001$) compared with men who were not infected with HPV. However, age, age at first receptive anal intercourse, and smoking status were not associated with ASILs ($P>.10$).

We constructed a multivariable logistic model to identify factors associated with ASILs. Because the number of male receptive anal sex partners in the previous 6 months was highly correlated with the number of male sex partners in the previous 6 months ($r>.8$), only the number of male receptive anal sex partners variable was used in the final multivariable model. The factors that showed independent, statistically significant associations with ASILs were having more than five receptive anal sex partners compared with fewer than two receptive anal sex partners during

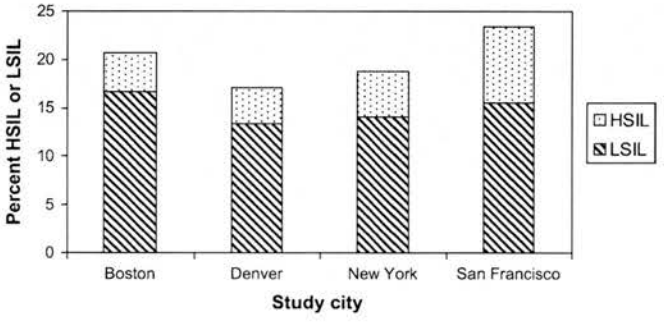

Fig. 2. Prevalence of anal squamous intraepithelial lesions by study city. The total height of each column represents the prevalence of anal squamous intraepithelial lesions. HSIL = high-grade squamous intraepithelial lesion; LSIL = low-grade squamous intraepithelial lesion.

Table 2. Predictors of any anal squamous intraepithelial lesions (ASILs)*

Characteristic	*N*	No. (%) with ASILs	Unadjusted		Multivariable†	
			OR (95% CI)	*P*‡	OR (95% CI)	*P*§
Demographics						
Age (per 10 y)			1.00 (0.86 to 1.2)	.98	0.92 (0.73 to 1.2)	.46
City						
San Francisco	330	86 (26)	1.00 (referent)		1.00 (referent)	
Boston	293	68 (23)	0.86 (0.59 to 1.2)	.41	0.90 (0.55 to 1.5)	.67
New York	263	56 (21)	0.77 (0.52 to 1.1)	.18	0.61 (0.37 to 1.03)	.07
Denver	227	46 (20)	0.72 (0.48 to 1.1)	.12	0.71 (0.41 to 1.2)	.22
Sexual behavior‖						
Age at first anal receptive intercourse (per 10 y)			1.2 (0.95 to 1.4)	.14	1.32 (1.01 to 1.7)	.045
No. of male sex partners						
<2	97	9 (9)	1.0 (referent)		ND	
2–5	268	62 (23)	2.1 (1.0 to 4.4)			
>5	796	185 (23)	3.1 (1.5 to 6.30)	<.001¶		
No. of male receptive anal sex partners						
<2	391	52 (13)	1.0 (referent)		1.0 (referent)	
2–5	303	59 (19)	1.6 (1.1 to 2.5)		1.2 (0.7 to 2.0)	
>5	546	142 (26)	2.4 (1.7 to 3.4)	<.001¶	1.8 (1.1 to 2.8)	.018¶
Substance use‖						
Smoker						
No	723	159 (22)	1.0 (referent)		1.00 (referent)	
Yes	222	53 (24)	1.1 (0.78 to 1.6)	.56	1.09 (0.69 to 1.7)	.71
Poppers						
No	783	140 (18)	1.0 (referent)		1.0 (referent)	
Yes	477	116 (24)	1.6 (1.2 to 2.1)	.002	1.4 (0.95 to 2.1)	.092
Cocaine						
No	1040	204 (20)	1.0 (referent)		1.0 (referent)	
Yes	221	52 (24)	1.4 (1.0 to 2.0)	.08	1.2 (0.73 to 2.0)	.46
Injection drugs						
Never	1009	231 (23)	1.0 (referent)		1.0 (referent)	
<2 times/month	75	16 (21)	0.9 (0.5 to 1.6)	.76	1.1 (0.55 to 2.4)	.73
≥2 times/month	9	6 (67)	6.7 (1.7 to 27)	.007	17 (1.2 to 223)	.035
HPV-related factors						
Anal HPV infection#						
No	428	29 (7)	1.0 (referent)		1.0 (referent)	
Yes	578	203 (35)	7.5 (4.9 to 11)	<.001	2.5 (1.3 to 4.9)	.008
No. of HPV types						
0	559	51 (9)	1.0 (referent)		1.0 (referent)	
1	255	83 (33)	4.8 (3.3 to 7.1)		2.7 (1.5 to 4.9)	
2	111	51 (46)	8.5 (5.3 to 14)		4.2 (2.1 to 8.3)	
3–4	65	34 (52)	11 (6.2 to 19)		6.2 (2.8 to 14)	
≥5	16	13 (81)	43 (12 to 157)	<.001¶	47 (5.3 to 411)	<.001¶
HPV-type infection						
None	464	34 (7)	1.0 (referent)			
Low-risk** only	160	69 (43)	9.6 (6.0 to 15)	<.001	ND	
High-risk†† only	146	42 (29)	5.1 (3.1 to 8.4)	<.001		
Low-risk and high-risk	105	65 (62)	21 (12 to 35)	<.001		

*Totals for *n* vary because of missing data. OR = odds ratio; CI = confidence interval; HPV = human papillomavirus; ND = not determined.
†Each variable was adjusted for other variables in column.
‡Two-sided *P* value from simple logistic regression.
§Two-sided *P* value from multiple logistic regression. Each variable was adjusted for other variables in column.
‖During the previous 6 months.
¶Test of linear trend across ordered groups specified.
#HPV DNA measured by polymerase chain reaction.
**Low-risk HPV types include types 6, 11, 53, 54, 55, 66, Pap 155, and Pap 291.
††High-risk (cancer-associated) HPV types include types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68, and 73.

the previous 6 months (OR = 1.8, 95% confidence interval [CI] = 1.1 to 2.8, $P = .018$), older age at first receptive anal intercourse (OR = 1.3 per 10-year increase in age, 95% CI = 1.0 to 1.7, $P = .045$), injection drug use two or more times per month compared with no use in the previous 6 months (OR = 17, 95% CI = 1.2 to 223, $P = .035$), PCR evidence of an anal HPV infection (OR = 2.5, 95% CI = 1.3 to 4.9, $P = .008$), and anal HPV infection with increasing numbers of HPV types ($P < .001$ for linear trend) (Table 2). Geographic location remained unassociated with the risk of ASILs in the adjusted model. Although neither age nor smoking status was statistically significantly associated with ASILs in univariate analyses, both factors were included in the final multivariable model because results of previous studies *(25)* had indicated that they are important risk factors for anal cancer. Neither factor was statistically significantly associated with the risk of ASILs in the final multivariable model.

Risk Factors for LSILs Compared With Risk Factors for HSILs

Factors that were associated with the risk of LSILs in unadjusted analyses were having more than five male receptive anal sex partners during the previous 6 months compared with having fewer than two receptive anal sex partners (OR = 2.7, 95% CI = 1.8 to 4.0, $P = .001$), older age at first receptive anal intercourse (OR = 1.3 per 10-year increase in age, 95% CI = 1.02 to 1.5, $P = .035$), use of poppers during the previous 6 months compared with no use (OR = 1.9, 95% CI = 1.4 to 2.6, $P<.001$), cocaine use during the previous 6 months compared with no use (OR = 1.3, 95% CI = 1.1 to 2.4, $P = .013$), use of injection drugs two or more times per month during the previous 6 months compared with no use (OR = 7.5, 95% CI = 1.8 to 31, $P = .006$), anal HPV infection (OR = 7.6, 95% CI = 4.7 to 12, $P<.001$), and anal HPV infection with increasing numbers of HPV types ($P<.001$ for linear trend) (Table 3). The risk of LSILs was also associated with infection with high-risk HPV types only (OR = 4.6, 95% CI = 2.6 to 8.3, $P<.001$), with infection with low-risk HPV types only (OR = 11, 95% CI = 6.5 to 18, $P<.001$), and with infection with both high-risk and low-risk HPV types (OR = 20, 95% CI = 11 to 36, $P<.001$) compared with men who were not infected with HPV. Factors that showed independent, statistically significant associations with LSILs in a multivariable model were having more than five male receptive anal sex partners during the previous 6 months compared with having fewer than two receptive anal sex partners (OR = 1.9, 95% CI = 1.1 to 3.2, $P = .028$), older age at first receptive anal intercourse (OR = 1.6 per 10-year age increase, 95% CI = 1.2 to 2.1, $P = .004$), injection drug use two or more times per month during the previous 6 months (OR = 19, 95% CI =1.3 to 277, $P = .03$), use of poppers during the previous 6 months (OR = 1.6, 95% CI = 1.1 to 2.5, $P = .03$), and anal HPV infection with increasing numbers of HPV types ($P<.001$ for linear trend) (Table 3). There was suggestive evidence that anal HPV infection, as detected by PCR, may be associated with the risk of LSILs (OR = 2.2, 95% CI = 0.98 to 5.1, $P = .056$). There was no statistically significant association between age or geographic location and the risk of LSILs in the adjusted model.

Factors that were statistically significantly associated with the risk of HSILs in unadjusted analyses included residence in San Francisco compared with residence in Boston (OR = 0.49, 95% CI = 0.25 to 0.96, $P = .04$) or Denver (OR = 0.46, 95% CI = 0.22 to 0.98, $P = .04$), an increasing number of male sex partners ($P = .047$ for linear trend); PCR evidence of an anal HPV infection (OR = 7.0, 95% CI = 3.3 to 15, $P<.001$), and anal HPV infection with increasing numbers of HPV types ($P<.001$ for linear trend). The risk of HSILs was also associated with infection with high-risk HPV types only (OR = 6.4, 95% CI = 2.7 to 15, $P<.001$), infection with both high-risk and low-risk HPV types (OR = 23, 95% CI = 9.6 to 53, $P<.001$), and infection with low-risk HPV types only (OR = 5.8, 95% CI = 2.3 to 14, $P<.001$), all compared with no HPV infection. A multivariable logistic model revealed that residence in New York compared with residence in San Francisco (OR = 0.41, 95% CI = 0.17 to 0.98, $P = .045$), PCR evidence of an anal HPV infection (OR = 3.2, 95% CI = 1.1 to 9.4, $P = .039$), and anal HPV infection with increasing numbers of HPV types ($P<.001$ for linear trend) were independently associated with the risk of HSILs (Table 3). There was no evidence (i.e., $P>.20$) for independent associations between age or older age at first receptive anal intercourse and the risk of HSILs. Using a polytomous logistic regression model to compare risk factors for LSILs with those for HSILs, we found that older age at first receptive anal intercourse had statistically significantly different associations with LSILs and HSILs ($P = .012$).

Discussion

Here we report the prevalence of ASILs among HIV-negative MSM, a population that is at an increased risk for the development of invasive anal cancer compared with the general population. We found that a high proportion of sexually active HIV-negative MSM over a wide age range had anal cancer precursor lesions as determined by anal cytology. Given that a previous study showed that anal cytology has only 50% sensitivity for diagnosing HSILs in HIV-negative MSM compared with the gold standard anal biopsy *(26)*, the true prevalence of HSILs is likely to be even higher than what we report here. The substantial prevalence of anal HSILs, in particular, places a high proportion of MSM at risk for anal cancer. The overall prevalence of ASILs by cytology was 20% and was similar across all age groups.

Several studies have demonstrated that, among women, the prevalence of cervical HPV infection and subsequent CSILs are strongly associated with age *(9,14)*. Women may acquire HPV infection at the onset of sexual activity, typically during late adolescence or during their early 20s. Most of these HPV infections are thought to resolve spontaneously. However, a small proportion of women have a persistent HPV infection that leads to CSILs, the incidence of which typically peaks 2–3 years after the initiation of sexual activity and then drops substantially among older women. An age-related decline in the prevalence of cervical HPV infection has been observed even among high-risk populations, such as female sex workers in Spain *(27)* and Mexico City *(28)*, as well as among sexually active inner-city women in New York *(29)*. By contrast, in the cohort of urban HIV-negative MSM that were recruited for this study, we previously demonstrated that anal HPV infection was not related to age, that anal HPV infection was found in 57% of participants, and that there was no age-related decrease in the prevalence of anal HPV infection *(15)*. Consistent with this finding is the lack of any age-related decrease in the prevalence of anal cytologic abnormalities, in sharp contrast with what has been observed for CSILs in women.

A number of factors could contribute to differences between the age associations of epidemiology of CSILs in women and that of ASILs in MSM. Although the cervix and the anus are histologically similar, they exist in different hormonal milieus, and the hormonal milieu of the cervix varies with age and menopausal status. However, a more likely explanation for the differences in age associations between the epidemiology of CSILs in women and that of ASILs in MSM is differences in the levels of sexual activity among women in the previous studies and the MSM in our study. The MSM in our study reported having a median of eight male sexual partners during the previous 6 months, a number that remained relatively constant for all age groups, but is substantially greater than the number of new sexual partners reported by most women older than 30 years. For example, behavioral data reported in previous cervical HPV epidemiologic studies show that sexual activity ranged from

greater than 50% of subjects having one lifetime sexual partner in a population-based study of cervical dysplasia in Costa Rica *(9)* to means of 2.2 sexual partners during the previous year among women 30 to 39 years old and 1.4 sexual partners during the previous year among women older than 40 years in a study of cervical HPV infection among a group of inner-city women in New York *(29)*. Studies of cervical HPV in sex workers in Mexico and Spain reported high numbers of sexual partners *(27,28)* but did not report the age-associated prevalence of cervical cytologic abnormalities and thus cannot be directly compared with our study.

Most of the men in our study had never been screened for ASILs before study entry. It was therefore interesting that the prevalence of LSILs and HSILs did not increase substantially with age because in the absence of screening and subsequent treatment of disease, we would expect an increasing proportion of men to develop ASILs in the older age groups. The fact that we did not observe a cumulative increase in the proportion of MSM with anal cytologic changes with increasing age suggests that many of the lesions that develop after HPV infection are transient. Consistent with this interpretation, we previously reported that the prevalence of anal HPV infection was consistent across all age groups *(15)* and was not cumulative. Our observation that older age at first receptive anal sex was associated with the risk of LSILs also suggests that many HPV lesions are transient in nature. If HPV lesions were transient, men who were younger at the time of their first sexual experience were probably exposed to HPV and then developed LSILs that regressed over time. Another, less likely, explanation for this finding would be a cohort effect: Older MSM could have been more sexually active and more intensely exposed to HPV than younger MSM in this prevalence study. The 20% prevalence of ASILs in the older men could therefore reflect a true age-related decrease in the prevalence of ASILs if that particular cohort of men had a substantially higher prevalence of ASILs during their 20s and 30s.

There was no difference in the overall prevalence of ASILs by geographic location. However, there was some evidence that location was a risk factor for HSILs, but not for LSILs. San Francisco had the highest prevalence of HSILs, at 8%, whereas the other sites each had an HSIL prevalence of approximately 4%. The reasons for this difference in prevalence, if it is a true difference, are not immediately apparent but may reflect differences in behaviors that were not captured by our questionnaire. This possible regional difference in the prevalence of HSILs should be explored in future population-based studies.

As has been seen previously *(30,31)*, anal HPV infection and the number of HPV types were important risk factors for anal lesions in our study. The strong association between anal HPV infection and ASILs that we found is consistent with the established role of HPV infection in the development of anal cancer *(4,25)*. Infection with multiple HPV types may be a marker of persistent disease and of the progression of LSILs to HSILs *(7)*. At present, there is little evidence that direct molecular interactions among different HPV types potentiate disease pathogenesis, but having multiple HPV types may represent an unmeasured factor(s) that is important in ASIL pathogenesis, such as attenuated HPV-specific immunity. Infection with a greater number of HPV types may also result from having a larger number of sexual encounters, which could potentially increase exposure to other unmeasured risk factors associated with anal intercourse, such as inflammation. Finally, infection with a greater number of HPV types may also reflect a greater number of lesions, which would increase the probability that an abnormality would be detected by anal cytology.

Previous studies have demonstrated that HPV type (i.e., high-risk versus low-risk) is associated with the type of lesions that later develop *(24,25)*. Compared with men in whom no HPV DNA was detected, men infected with low-risk HPV types only as well as men infected with high-risk HPV types only had an increased risk of LSILs. Similarly, men who were infected with low-risk HPV types only or with high-risk HPV types only had an increased risk of HSILs compared with men with no HPV infection. Given that earlier studies reported that only high-risk HPV types are closely linked with the development of invasive cervical and anal cancers [reviewed in *(25)*], it is interesting to note that low-risk HPV types were also associated with the risk of HSILs in our study population. However, we found that infection with both high-risk and low-risk HPV types was more strongly associated with either LSILs or HSILs than infection with either high-risk or low-risk HPV types only, consistent with our observation that an increasing number of HPV types is an important risk factor for ASILs. Longitudinal studies that examine the temporal association between type-specific HPV infection and progression of ASILs are needed to clarify the relationship between infection with specific HPV types and the risk of incident and persistent HSILs.

Behavioral determinants that were strongly associated with the risk of ASILs included the number of male sex partners during the previous 6 months and the number of receptive anal sex partners during the previous 6 months. These behavioral risk factors for ASILs are not surprising, given the strong evidence for the role of sexual transmission of HPV as a necessary step for development of anal cancer precursor lesions *(4)*. We also found evidence for the independent association of injection drug use and use of poppers with the risk of ASILs. A high prevalence of ASILs and anal HPV infection was previously reported among HIV-infected injection drug users who denied having anal intercourse *(32)*. The roles of injection drug use and the use of poppers in the pathogenesis of ASILs is unclear; however, the use of such substances may be a proxy for partner selection or for risk behaviors that were not captured in our structured questionnaire.

Strengths of our study include the large number, geographic diversity, and wide age spectrum of the participants. One limitation of our study is that study participants of all ages remained sexually active, with multiple partners. Thus, it is possible that our results are not generalizable to all HIV-negative MSM. However, data collected by the probability-based Urban Men's Health Study *(33)* on the sexual behavior of predominantly HIV-negative MSM in four cities (San Francisco, Los Angeles, New York, and Chicago) suggest that the men in our study are highly representative of urban MSM with respect to age at first anal intercourse, any history of anal sex, and history of anal sex during the previous year. Multiple recent cross-sectional surveys of mostly HIV-uninfected MSM in San Francisco *(34)*, London *(35)*, and Amsterdam *(36)* also report sexual behavior similar to that of the men in our study. Thus, although participants in our study are likely to be representative of many urban HIV-negative MSM, future studies that use probability-based sampling are needed before these findings can be generalized to all MSM, particularly to those who have fewer sexual partners than reported by participants in this study. Another possible limitation of our study is the fact that only one pathologist reviewed all of the cytology.

Table 3 (continued). Predictors of anal low-grade squamous intraepithelial lesions (LSILs) and high-grade squamous intraepithelial lesions (HSILs)

Table 3. Predictors of anal low-grade squamous intraepithelial lesions (LSILs) and high-grade squamous intraepithelial lesions (HSILs)*

Characteristic	*N*	Predictors of LSIL					Predictors of HSIL				
		No. (%) with LSILs	Unadjusted		Multivariable†		No. (%) with HSILs	Unadjusted		Multivariable†	
			OR (95% CI)	*P*‡	OR (95% CI)	*P*§		OR (95% CI)	*P*‡	OR (95% CI)	*P*§
Demographics											
Age (per 10 y)			0.94 (0.8 to 1.1)	.46	0.82 (0.63 to 1.1)	.15		1.2 (0.92 to 1.5)	.20	1.2 (0.8 to 1.7)	.40
City											
San Francisco	330	57 (17)	1.00 (referent)		1.00 (referent)		29 (9)	1.00 (referent)		1.00 (referent)	
Boston	293	55 (19)	1.05 (0.69 to 1.6)	.83	0.99 (0.57 to 1.7)	.99	13 (4)	0.49 (0.25 to 0.96)	.04	0.72 (0.32 to 1.6)	.44
New York	263	42 (16)	0.87 (0.56 to 1.3)	.53	0.72 (0.40 to 1.3)	.26	14 (5)	0.57 (0.29 to 1.1)	.10	0.41 (0.17 to 0.98)	.45
Denver	227	36 (16)	0.85 (0.54 to 1.3)	.49	0.82 (0.44 to 1.5)	.53	10 (4)	0.46 (0.22 to 0.98)	.04	0.47 (0.17 to 1.2)	.13
Sexual behavior‖											
Age at first anal receptive intercourse (per 10 y)			1.3 (1.02 to 1.5)	.035	1.6 (1.2 to 2.1)	.004		0.88 (0.6 to 1.3)	.52		
No. of male sex partners											
<2	97	8 (8)	1.00 (referent)		ND		1 (1)	1.00 (referent)		ND	
2–5	268	43 (16)	1.6 (0.73 to 3.6)				19 (7)	5.7 (0.75 to 43)	.047¶		
>5	796	139 (17)	2.6 (1.2 to 5.5)	.001¶			46 (6)	6.9 (0.94 to 51)			
No of male receptive anal sex partners											
<2	391	36 (9)	1.00 (referent)		1.00 (referent)		16 (4)	1.00 (referent)		1.00 (referent)	
2–5	303	43 (14)	1.7 (1.1 to 2.8)		1.3 (0.70 to 2.3)		16 (5)	1.4 (0.71 to 3.0)		0.95 (0.38 to 2.4)	
>5	546	109 (20)	2.7 (1.8 to 4.0)	<.001¶	1.9 (1.1 to 3.2)	.028¶	33 (6)	1.8 (0.98 to 3.4)	.059¶	1.5 (0.71 to 3.3)	.21¶
Substance use‖											
Smoker											
No	723	118 (16)	1.00 (referent)		1.00 (referent)		41 (6)	1.00 (referent)		1.00 (referent)	
Yes	222	39 (18)	1.1 (0.74 to 1.6)	.63	1.1 (0.65 to 1.8)	.79	14 (6)	1.1 (0.61 to 2.1)	.69	1.1 (0.52 to 2.3)	.81
Poppers											
No	783	96 (12)	1.00 (referent)		1.00 (referent)		44 (6)	1.00 (referent)		1.00 (referent)	
Yes	477	94 (20)	1.9 (1.4 to 2.6)	<.001	1.6 (1.1 to 2.5)	.03	22 (5)	0.95 (0.56 to 1.6)	.85	0.93 (0.48 to 1.8)	.83
Cocaine											
No	1040	146 (14)	1.00 (referent)		1.00 (referent)		58 (6)	1.00 (referent)		1.00 (referent)	
Yes	221	44 (20)	1.6 (1.1 to 2.4)	.013	1.3 (0.77 to 2.3)	.31	8 (4)	0.74 (0.35 to 1.6)	.45	0.83 (0.32 to 2.1)	.70

(Table continues)

Moreover, any potential under- or overdiagnosis of ASILs would probably affect only our prevalence estimates, not the estimates of associations with potential risk factors. However, the pathologist who interpreted the slides for this study participated in a study of interobserver variability in anal cytology, which showed moderately good interobserver agreement (κ = 0.55–0.88) for anal cytology between experienced pathologists (T. Darragh, personal communication).

Our data indicate that a high proportion of sexually active HIV-negative MSM in all age groups have ASILs, including HSILs. The high prevalence of ASILs may have direct implications for anal cancer screening. Given the similarities between cervical cancer and anal cancer and given the success of cervical cancer screening in reducing mortality, Palefsky et al. *(26)* have proposed an anal cytology screening protocol to identify women and men who may have anal cancer precursor lesions. Results of cost-effectiveness analyses indicate that a reasonable screening interval is 2–3 years for HIV-negative MSM *(37)*. It has been further suggested that patients who have an abnormal anal cytology (i.e., Pap) test should undergo high-resolution anoscopy, which uses magnification and characteristic changes in appearance of diseased tissue after the application of acetic acid to identify lesions that should undergo biopsy and staging. Various treatment options that are based on the grade, size, and location of these lesions have been described *(38)*. Given that anal cancer occurs only rarely in HIV-negative men aged younger than 40 years, one approach would be to initiate screening only among men older than 40 years. In addition, although the role of anal HPV testing to identify MSM who are at risk for anal cancer has not been fully explored, its primary use may rest in its negative predictive value.

Our study illustrates that the epidemiology of anal cancer precursor lesions in sexually active HIV-negative MSM is different from that of cervical cancer precursor lesions in sexually active women. The high prevalence of HPV-related anal disease at all age groups reflects a population that continues to have a large number of new sexual exposures over many decades of life. In contrast, the prevalence of HPV-related cervical disease in women is lower and declines with age. Extrapolations from the cervical cancer prevention literature to anal cancer screening cannot be made blindly. Specific knowledge of anal HPV and ASIL epidemiology is even more relevant today as advances continue to be made in the therapy of ASILs *(39)*, as well as in HPV-specific therapeutic *(40)* and prophylactic *(41)* vaccines.

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Notes

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APPENDIX 8

Human papillomavirus is associated with HIV acquisition: the EXPLORE study.

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Introduction: Human papillomavirus (HPV) is a common sexually transmitted agent and can cause anal intraepithelial neoplasia (AIN). Abnormal anal cytologic changes associated with HPV infection include atypical squamous cells (ASC), low-grade squamous intraepithelial lesions (SIL) and high-grade SIL. We hypothesized that AIN can enhance susceptibility to HIV infection because of increased microvasculature, bleeding and recruitment of CD4+ and dendritic cells. We studied the association between the detection of anal HPV or abnormal anal cytology and HIV acquisition.

Methods: This multisite prospective study recruited 1409 HIV-negative sexually active men who have sex with men. During each twice-yearly follow-up visit, we assessed anal HPV infection status by polymerase chain reaction, obtained anal cytology and behavioral data, and conducted HIV antibody testing.

Results: Of 1409 participants followed for up to 36 months, 51 HIV-seroconverted. Of the HIV seroconverters, 81% had anal HPV, and 43% had abnormal anal cytology

at the time of HIV seroconversion. The median number of HPV types in HPV-infected HIV-seroconverters was 2 (IQR, 1-3) at the time of HIV-seroconversion. In univariate analyses, there was evidence ($P<0.05$) for an association between anal HPV infection (HR 2.7, 95% CI 1.0-7.3) and detection of 3 HPV types (HR 3.7, 95% CI 1.4-10.3) in predicting HIV seroconversion, and moderate evidence ($P<0.10$) for ASC (HR 2.4, 95% CI 0.9-6.1). After adjustment for sexual activity, substance use and demographics, there was evidence ($P<0.05$) for the effect of 3 HPV types isolated (HR 3.3, 95% CI 1.1-9.9) and ASC (HR 2.8, 95% CI 1.1-7.8). **Conclusions:** Anal HPV and ASC on anal cytology are independently associated with HIV acquisition. Prospective studies that incorporate high-resolution anoscopy for more sensitive visual and histologic identification of AIN are needed to extend these cytologic findings. Identification of HPV and HPV-associated lesions may improve assessment of HIV transmission risk.